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ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DPN	Diphosphopyridine nucleotide
EDTA	Ethylenediaminetetra-acetate
FMN	Flavin mononucleotide
FAD	Flavin–adenine dinucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
TPN	Triphosphopyridine nucleotide
tris	2-Amino-2-hydroxymethylpropane-1:3-diol

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Changes in the Chemical Composition of a Cambial Cell during its Differentiation into Xylem and Phloem Tissue in Trees

1. MAIN COMPONENTS

By J. P. THORNBER and D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge

(*Received* 16 *March* 1961)

Investigations of the changes in the composition of the plant cell during its development can be used as a basis for the study of the mechanism by which the cellular materials are synthesized during secondary thickening of the cell wall. The formation of xylem and phloem tissue in trees has been studied in this way (Allsopp & Misra, 1940; Anderson & Pigman, 1947; Anderson, Wise & Ratliff, 1954; Meier & Wilkie, 1959; Stewart, Amos & Harvey, 1953; Sultze, 1957), and similar investigations of the development of the cell in non-woody tissues have also been made (Fuller, 1958; Jensen & Ashton, 1960; Jermyn & Isherwood, 1956; Whistler & Young, 1960; Wirth, 1946).

However, the percentage composition of whole tissue compared at different stages of growth gives little indication of the changes occurring in a single cell during its development. This information can only be obtained if the weight, in addition to the percentage composition, of a single cell is measured during its differentiation. This paper describes such changes during the development of xylem cells from cambial cells in four species of trees. The percentage composition of the phloem in each species is also reported.

EXPERIMENTAL

Materials

Material for phloem, sapwood and heartwood analyses. Samples were taken from a 30-year old birch (*Betula platyphylla*), a 15-year-old pine (*Pinus ponderosa*), and a 50-year-old sycamore (*Acer pseudoplatanus*) that are growing in the Botanical Gardens, Cambridge, and from a 15-year-old ash (*Fraxinus elatior*) that is growing in Madingley Wood, Cambridge.

A Mattson's increment borer (Pressler borer, J. H. Steward Ltd., Strand, London, W.C. 2) was used to remove four plugs about 3 cm. long from each tree during the period 16–20 November 1959. The samples, which were taken at a height of 4 ft. 3 in. from the ground, were immediately placed in glass-stoppered tubes and frozen to $-55°$ (solid CO_2 and acetone). The samples were cut with a razor blade into sections corresponding to phloem, sapwood and heartwood, the sycamore phloem being further divided into inner and outer portions (Fig. 1).

Material for cambial tissue analyses. Cambial tissue was obtained from ash (*F. elatior*) and sycamore (*A. pseudoplatanus*) saplings (4 years old) grown in Madingley Wood, Cambridge, and from pine (*Pinus sylvestris*) and birch (*Betula* sp.) saplings (4–8 years old) grown in Mildenhall Woods, Thetford. The material was collected during the period 27 July–4 August 1959.

After the outer layers had been stripped from the saplings the soft cambial tissue beneath was gently scraped off and immediately frozen to $-55°$ (solid CO_2 and acetone). Microscopical examination of the material obtained confirmed that it was made up almost completely of cambial cells.

Analytical methods

Drying of samples and preparations. The frozen samples were dried at 100° over P_2O_5 at 0·5 mm. Hg. All pectic substances, holocellulose, hemicellulose and α-cellulose preparations were dried at room temperature over P_2O_5 at 0·1 mm. Hg. Water-extracted material, ethanol- and benzene-extracted material, lignin and material from which the pectic substances had been removed were dried to constant weight in an oven at 105°.

Water content. The decrease in weight of a fresh sample was found after it had been heated at 100° for 3 hr. at 0·5 mm. Hg over P_2O_5.

Ash. This was obtained from a portion of the dried material which had been maintained at dull red heat (500–600°) in a Pt boat in a stream of clean dry air for 2 hr.

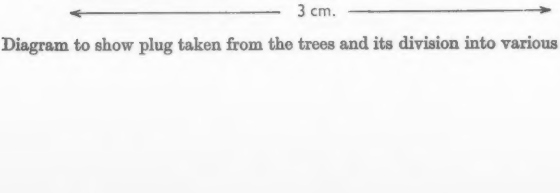

Fig. 1. Diagram to show plug taken from the trees and its division into various regions.

Total nitrogen. This was determined by micro-Kjeldahl digestion (Chibnall, Rees & Williams, 1943) followed by distillation and titration and expressed as protein after multiplication by the factor 6·25.

Total phosphorus. This was determined according to Fiske & Subbarow (1925).

Fractionation of the samples (Fig. 2)

Water-soluble material. About 150 mg. of dried material, which had been chopped and passed through a 16-mesh sieve, was extracted three times with distilled water at 100° for 3 hr. After filtration the extracted material was dried and weighed. The extractives were freeze-dried and weighed.

Ethanol- and benzene-soluble material. The water-extracted material was further extracted with ethanol–benzene (1:2, v/v) at 70° for 6 hr. The extractive-free material was dried and weighed. The ethanol and benzene were allowed to evaporate from the extractives and the residue was weighed.

Lignin. Approximately one-quarter of the already extracted material was used for the estimation of lignin by the method of Ritter, Seborg & Mitchell (1932). After treatment with H_2SO_4, the residue was centrifuged and thoroughly washed, by suspension and recentrifuging, with water. The dried residue was weighed.

Pectic substance. To the remainder of the already extracted material was added 0·2 M-EDTA (disodium salt), pH 4·5 (5 ml.). The mixture was shaken in a glass-stoppered tube by a Microid flask shaker (Griffin and George Ltd., Wembley, Middlesex) at room temperature for 4 hr. After filtration the EDTA solution was dialysed overnight against water. Ethanol was added to the dialysed solution until its concentration was over 80%, and the precipitate that formed was allowed to settle for 24 hr. This precipitate was centrifuged, washed with ethanol, dried and weighed. The dried residual material was weighed.

Holocellulose. The EDTA-extracted material was suspended in 5 ml. of water in a glass-stoppered tube, in a water bath at 75° for 2 hr. Two drops of acetic acid and 30 mg. of $NaClO_2$ were added and after 1 hr. these additions were repeated (Wise, Murphy & D'Addieco, 1946). The holocellulose was washed with water, acetone and ether and the dried residue was weighed.

Hemicellulose and α-cellulose. The holocellulose was fractionated by successive extractions with 5 and 24% (w/v) KOH solution under N_2 at 25° for a total of 4 hr. (Wise *et al.* 1946.) The residue was centrifuged and thoroughly washed with water by suspension and recentrifuging. The washings and the alkaline extracts were combined and neutralized at 4° with cooled acetic acid. The neutralized solution was dialysed for 24 hr. against running tap water followed by distilled water, and the hemicellulose precipitated in the same manner as that described for the pectic substances. The dried material was weighed. The residual α-cellulose was washed with water, acetone and ether, dried and weighed. The yield of α-cellulose was corrected for the amount of ash which it contained.

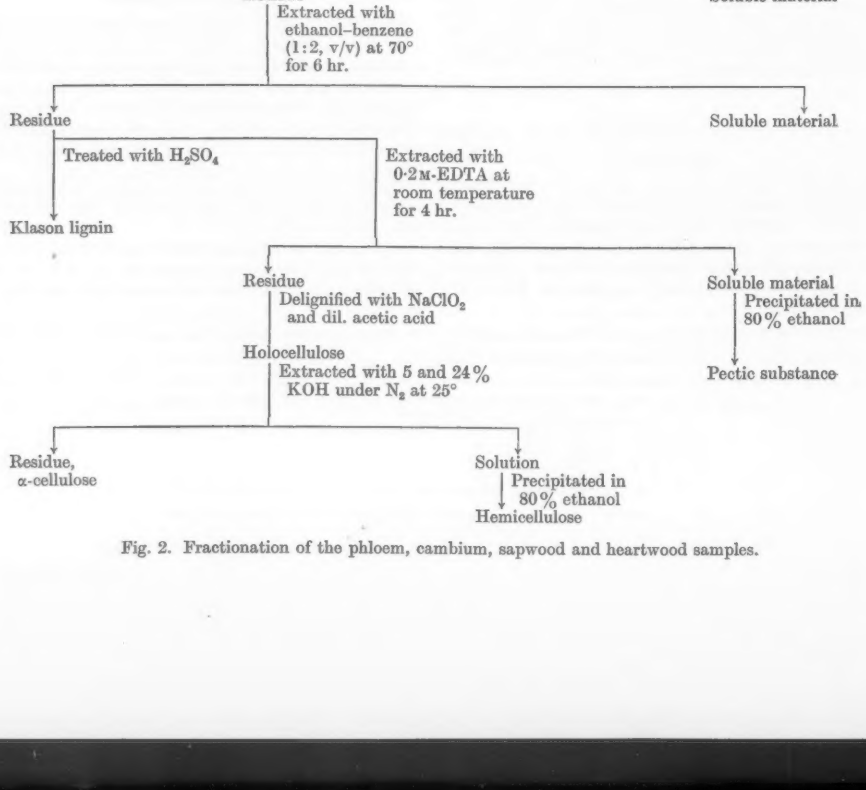

Fig. 2. Fractionation of the phloem, cambium, sapwood and heartwood samples.

Determination of the weight of a single cell in the cambial, sapwood and heartwood regions of each tree

Sapwood and heartwood. Sapwood and heartwood samples from each tree were extracted with water and ethanol–benzene (1:2, v/v) and then fixed in a dilute chromic acid fixative (Johansen, 1951), dehydrated and embedded in wax. A sledge microtome was used to cut transverse sections approx. 25 μ thick. The wax was removed from five sections from each region with xylene and after drying they were weighed (five sections weighed approx. 1 mg.). The sections were stained in 1% (w/v) safranin in 48% ethanol and mounted on a microscope slide. Each transverse section was projected through a light-microscope on a screen, and the total number of cells (about 1000) was counted in a measured area (0·25 mm.^{2}). This was repeated three times for different areas of each section. The total area of each transverse section was measured (approx. 13 mm.^{2}) and hence the total number of cells in the five sections could be calculated.

The transverse sections were removed from their mounts, washed in xylene and re-embedded in wax. Longitudinal sections were cut from these blocks, and the width of the specimen, which was measured on a screen after projection through a microscope, gave the thickness of the original transverse section.

The length of a single cell was determined from longitudinal sections (approx. 90 μ thick) which were cut from the original blocks. Sections were taken across one annual growth ring. They were dewaxed in xylene and delignified by the method of Wise *et al.* (1946). The sections were gently ground in a mortar with 24% (w/v) KOH (1 ml.); after 1 min. 10N-H_2SO_4 was added until the mixture was neutral (litmus). The fibres were spun down, washed with water and recentrifuged. They were stained with aqueous 0·5% (w/v) methylene blue, centrifuged and mounted in glycerol on a glass slide. The length of approx. 30 cells was measured in each sample. Microscopic examination of the tissue throughout this treatment showed that no appreciable alteration in the dimensions of the cell occurred.

Distances on the screen for the measurement of length, area and width of the section were calibrated from a standard graticule (Graticules Ltd., London, E.C. 1). The weight of a single cell was given by:

$$\frac{\text{Wt. of the five sections} \times \text{length of cell}}{\text{Total no. of cells in sections} \times \text{thickness of section}}.$$

Standard deviations were determined for the total number of cells in a known area and for the length of a cell (Table 2). The standard deviation of the weight of a cell was calculated (Table 1).

Cambium. Cambial tissue from each tree was extracted with water and ethanol–benzene (1:2, v/v). The dried extracted material was weighed and suspended in 0·02M-EDTA (30 ml.) in a polythene beaker which was placed in water in an ultrasonic shaker (Disontegrator system forty; Ultrasonics Industries Inc., Albertson, L. I, New York). A little detergent (Teepol) was added to the water outside the beaker in order to obtain more efficient propagation of the ultrasonic vibrations into the suspension. After shaking for 1 hr. at room temperature, 75% of the material was present as single cells. This mixture was centrifuged, and the cells were stained with aqueous 0·5% (w/v) methylene blue and recentrifuged. The cells were made up to a volume of 1 ml. with water.

A known volume of the cells (5 μl.) was placed on a microscope slide and the total number of cells and fractions of cells in this sample were counted. This was repeated eight times. The weight of a single cell was then calculated (Table 1). The length of a cambial initial was measured (Table 2).

Table 1. *Weight of an extractive-free cell*

Values are given in μmg.±S.D. with number of observations in parentheses.

	Region		
	Cambium	Sapwood	Heartwood
Ash	21·3±0·8 (8)	155±22 (28)	173±18 (28)
Birch	25·9±1·6 (8)	117±14 (30)	125±16 (24)
Sycamore	15·3±1·3 (8)	157±19 (25)	171±28 (30)
Pine	98·0±7·8 (8)	503±79 (28)	430±61 (40)

Table 2. *Length of a cell in each region of each tree*

Values are given in mm.±S.D. with number of observations in parentheses.

	Region		
	Cambium	Sapwood	Heartwood
Ash	0·71±0·12 (8)	0·98±0·14 (28)	1·08±0·11 (28)
Birch	0·63±0·10 (8)	0·92±0·14 (24)	1·01±0·13 (24)
Sycamore	0·52±0·08 (8)	0·72±0·09 (25)	0·68±0·11 (30)
Pine	0·80±0·13 (8)	1·47±0·23 (28)	1·28±0·21 (40)

RESULTS

In order that a comparison could be made of the development of the cell in different types of trees, four species were used: *F. elatior*, a ring porous angiosperm, *B. platyphylla* and *A. pseudoplatanus*, diffuse porous angiosperms, and *P. ponderosa*, a gymnosperm. Small quantities of material were taken for the determination of the composition of each region, since this work formed part of an investigation into the seasonal variation in the composition of each region of each tree. Removal of larger quantities over a yearly period would have seriously damaged the trees. The results presented in Tables 3 and 4 are those from samples taken during 1959; material taken at corresponding dates during 1960 gave the same analyses.

In our investigation material from the phloem, cambial, sapwood and heartwood regions of each tree was fractionated into more components than in any similar investigation since that of Stewart *et al.* (1953).

Weight and length of a single extractive-free cell in the cambial, sapwood and heartwood region of each tree. The weights of single cells are shown in Table 1. Cells from all regions of the gymnosperm weighed more than the corresponding cells of the angiosperms, and during differentiation of a cambial cell

into xylem the increase in weight was greatest in the pine and least in the birch. In the angiosperms the mean weight of the sapwood cell was less than that of the heartwood cell, but in pine this was not so.

The lengths of single cells are given in Table 2. In order to account for the variation in length of the cell across an annual growth ring (Bisset, Dadswell & Amos, 1950), the lengths were measured from sections representative of one complete growth

Table 3. *Composition of the samples taken from each tree*

Values are expressed as a percentage of the whole dry material. Water content is expressed as a percentage of the whole fresh material.

		Region of plug			
		Phloem	Cambium	Sapwood	Heartwood
Ash	Water content	45·4	83·8	28·4	28·9
	Ash	7·6	10·6	0·5	0·6
	Protein	1·0	9·4	0·5	0·5
	Phosphorus	0·55	0·30	0·10	0·05
	Water-soluble material	35·1	65·3	9·1	6·8
	Ethanol–benzene-soluble material	2·5	1·9	2·1	0·2
Birch	Water content	38·2	86·7	49·8	48·6
	Ash	4·9	8·0	0·3	0·2
	Protein	3·5	10·6	0·6	0·5
	Phosphorus	0·11	0·24	0·08	0·04
	Water-soluble material	18·5	49·6	11·6	3·5
	Ethanol–benzene-soluble material	8·3	3·7	1·4	1·0
		Outer / Inner			
Sycamore	Water content	38·0 / 51·5	90·0	43·6	43·9
	Ash	9·8 / 7·3	17·9	0·6	0·5
	Protein	2·0 / 9·9	17·2	1·2	0·8
	Phosphorus	0·02 / 0·05	1·1	0·06	0·03
	Water-soluble material	9·8 / 15·4	65·6	8·6	7·2
	Ethanol–benzene-soluble material	1·4 / 1·6	3·7	1·2	0·5
Pine	Water content	57·6	84·9	42·2	48·0
	Ash	1·3	3·1	0·2	0·2
	Protein	3·1	7·4	0·6	0·3
	Phosphorus	0·07	0·15	0·06	0·03
	Water-soluble material	34·7	61·0	5·7	4·3
	Ethanol–benzene-soluble material	9·0	5·3	2·5	3·3

Table 4. *Composition of the samples taken from each tree*

Values are expressed as a percentage of the material already extracted with water and ethanol–benzene (1:2, v/v).

		Region of plug			
	Fraction	Phloem	Cambium	Sapwood	Heartwood
Ash	Pectic substances	18·0	6·6	1·4	0·5
	Lignin	23·4	10·0	22·8	22·5
	α-Cellulose	28·5	42·6	42·4	44·4
	Hemicellulose	30·2	40·8	32·9	29·8
Birch	Pectic substances	4·4	18·0	2·6	0·3
	Lignin	53·1	10·0	23·0	22·5
	α-Cellulose	22·3	35·2	40·0	43·4
	Hemicellulose	19·2	36·5	35·0	33·8
		Outer / Inner			
Sycamore	Pectic substances	3·1 / 6·6	15·0	3·8	1·3
	Lignin	46·9 / 22·6	2·5	25·3	24·3
	α-Cellulose	29·0 / 45·0	36·7	40·0	43·0
	Hemicellulose	21·3 / 25·8	45·0	31·5	31·4
Pine	Pectic substances	10·0	8·5	1·3	1·0
	Lignin	52·8	20·0	30·4	29·5
	α-Cellulose	22·1	36·1	45·0	43·4
	Hemicellulose	15·3	35·0	23·0	25·0

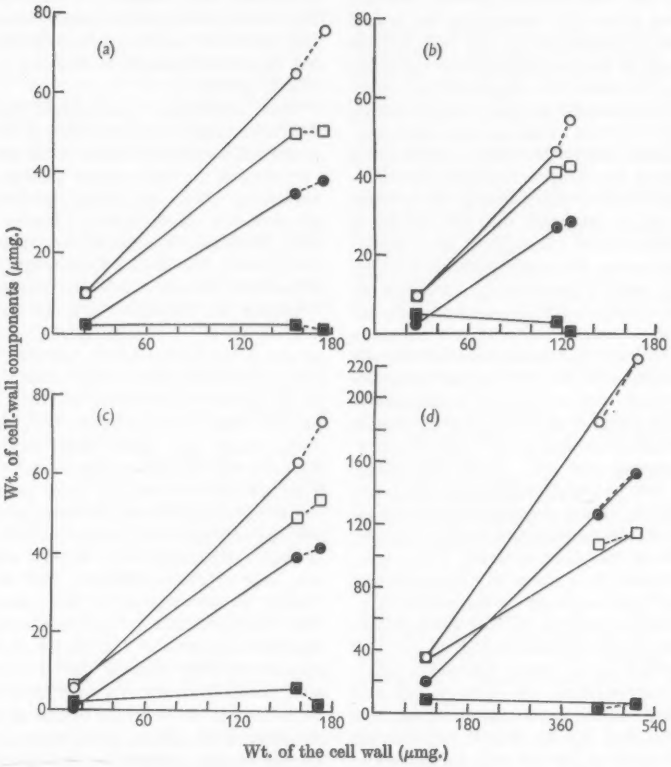

Fig. 3. Weight of each cell-wall component at different stages of development. (*a*), *F. elatior*; (*b*), *B. platyphylla*; (*c*) *A. pseudoplatanus*; (*d*), *P. ponderosa*. ■, Pectic substances; ●, lignin; ○, α-cellulose; □, hemicellulose. ——, Cambial cell to sapwood development; - - - - -, sapwood cell to heartwood development.

ring. The mean lengths of the cells in each region of the gymnosperm were longer than those in the corresponding regions of the angiosperms. The mean length of a cell increased with age in each species except in the gymnosperm, where a sapwood cell appeared longer than a heartwood cell.

Percentage composition of each region of each tree. The results are given in Tables 3 and 4. In order to compare the composition of the cell walls of the cambium with those of the other regions it is necessary to use the analytical data of the extractive-free material (Table 4), since the total composition included the material of the cell contents in addition to the cell-wall components.

Changes in weight of each cell-wall component during the differentiation of a cambial cell into a xylem cell. This may be calculated from the information in Tables 1 and 4. The increase in each component of the cell wall during secondary thickening is illustrated in Fig. 3.

DISCUSSION

It appeared from previous work that secondary thickening of a cambial cell involved the deposition of large amounts of lignin, hemicellulose and α-cellulose, and that little or no further addition was made to the pectic substances. These conclusions were based on the results of histochemical investigations and from comparisons of the percentage composition of the tissue at different stages of development (Allsopp & Misra, 1940; Anderson & Pigman, 1947; Asunmaa & Lange, 1954; Lange, 1954). Allsopp & Misra (1940) stated that the change in relative

composition of the tissue during growth does not give information about the changes in the actual amounts of each component of the cell. Fuller (1958) and Jensen & Ashton (1960) determined the weight of each component in a single cell and found the changes which occurred in their weights during the development of a cell in tissues from root tips. In tissues of wood, Meier & Wilkie (1959) have attempted to show the relative increases in the individual polysaccharide components of the cell wall during thickening by assuming that the weight of the wall was proportional throughout its development to its thickness. No assessment of the possible change in weight because of alteration in length was made. Sultze (1957) assumed that the total amount of araban in the wall did not vary from the initial value found in the cambial cell, and thus the composition of the developing tissue related to this araban content enabled a calculation of the percentage increase of the other components for a fixed amount of araban to be directly correlated to the increase per cell. Again this latter assumption is not entirely satisfactory and can only be justified by direct measurements such as those reported in this investigation (Fig. 3) and that of Thornber & Northcote (1961).

Our results showed that secondary thickening of the cambial cell wall occurred by formation of α-cellulose, hemicellulose and lignin with little change in weight of the pectic substances (Fig. 3). This thickening resulted more from deposition of α-cellulose than hemicellulose, the cambial cell wall having contained equal amounts of these two fractions (Table 4 and Fig. 3). In the angiosperms the increase in weight of the cell wall during secondary thickening which varied for the individual species (birch, 350%; ash, 640%; and sycamore, 930%) was represented by the same relative increase of each component in all three species: α-cellulose, 40–43%; hemicellulose, 30–34%; and lignin, 25–27%. However, in the gymnosperm the increase (410%) was made up by α-cellulose, 47%; hemicellulose, 20%; and lignin 33%.

The α-cellulose fraction of the pine contained a glucomannan (Meier, 1958), which is generally considered to be a component of the hemicellulose. If the amount of the glucomannan is subtracted from the α-cellulose and included in the hemicellulose, then the only difference between the secondary thickening in the angiosperms and the gymnosperm is that in the latter relatively more lignin is formed.

The weight of lignin laid down during the formation of a sapwood cell was very large when expressed as a percentage of the amount present in the cambial cell (680–9300%). This showed that its deposition occurred almost exclusively during secondary thickening. It was also found that the ratio of the increase in weight of lignin to that of α-cellulose was constant in each species (Fig. 3). This fact lends support to the hypothesis that cellulose acts as a catalytic matrix for the formation of the lignin component (Goodman & Siegel, 1959; Siegel, 1957).

The percentage composition and the carbohydrate composition (Thornber & Northcote, 1961) of the cell-wall components of the cambial cell were very similar in each species (Table 4) and closely resembled those of other cambial tissues and primary cell walls (Bishop, Bayley & Setterfield, 1958; Kivilaan, Beaman & Bandurski, 1959; Northcote, 1958). It was only during differentiation that differences were seen between the species.

During the transition of a sapwood cell into the heartwood in the angiosperms, an increase occurred in the weight of the cell (ash, 11·6%; birch, 6·8% and sycamore, 8·9%). This was largely due to the greater deposition of the α-cellulose component (ash, 71%; birch, 77% and sycamore, 62%) than the lignin and hemicellulose components. In the gymnosperm, the mean weight of a heartwood cell was less than that of a sapwood cell and during the transformation of the sapwood cell into the heartwood a loss occurred in the weight of each component (Fig. 3), the largest of which was that of the α-cellulose. The most significant change in the amount of any component during this transformation in each species was the almost complete absence of the pectic substances in the heartwood. This may be due to a removal of this component from the cell wall, or it may be due to its becoming more firmly bound in the cell wall of the heartwood. If this latter reason is correct there should be an increase in the carbohydrate composition of the other fractions to account for the pectic substances (Thornber & Northcote, 1961).

The percentage composition of the cell wall in the phloem is reported for each species (Tables 3 and 4). These cell walls had a higher percentage of pectic substances than those in the xylem, and in the ash and pine phloem the content of pectic substances was even greater than in the cambial region. The major component in this region was represented by substances which were insoluble in 72% (w/w) sulphuric acid, and the relative amounts of these substances increased with the age of the tissue (Table 4). Because of the large amounts of material other than lignin which may give rise to insoluble products with 72% sulphuric acid in the phloem region (Stewart *et al.* 1953), the values for the Klason lignin obtained for this region are not directly comparable with those for the xylem.

The percentage of materials usually associated with the contents of the cell rather than the cell wall are high in the cambial region, as might be expected. The percentage of these materials is less in the sapwood and even smaller in the heartwood

(Table 3). A single cell in any region of the gymnosperm was five times greater in weight than in the corresponding regions in the angiosperms and contained proportionally smaller percentages of ash, protein and phosphorus. In the xylem of the ash, the amount of water (30% of the fresh weight) was much smaller than in the xylem of the other trees; Allsopp & Misra (1940) have also reported this.

SUMMARY

1. A quantitative determination has been made of the changes that occurred in the composition of a cambial cell during its differentiation and subsequent maturation in four species of tree, *Acer pseudoplatanus*, *Betula platyphylla*, *Fraxinus elatior* and *Pinus ponderosa*.

2. The weight of α-cellulose, hemicellulose, lignin and pectic substances in the cell wall of a single cell in the cambium, sapwood and heartwood of each tree has been determined.

3. Comparison of the composition of these regions showed that secondary thickening of the cambial cell involved the deposition of α-cellulose, hemicellulose and lignin. This thickening resulted more from α-cellulose than hemicellulose, the cambial cell having equal amounts of each.

4. The amount of pectic substances per cell remained constant during the formation of the sapwood cell, but in the heartwood cell there was an almost complete absence of this fraction.

5. The composition of the cambial cell in each species was very similar.

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Biochem. J. (1961) **81**, 455

Changes in the Chemical Composition of a Cambial Cell during its Differentiation into Xylem and Phloem Tissue in Trees

2. CARBOHYDRATE CONSTITUENTS OF EACH MAIN COMPONENT

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Thornber & Northcote (1961) have reported the changes in the amounts of the pectic substances, lignin, hemicellulose and α-cellulose components of a cambial cell wall of four species of trees during its differentiation into a sapwood cell and its subsequent maturation into a heartwood cell. This paper reports the changes in the carbohydrate composition of the pectic substances, hemicellulose and α-cellulose components of a cambial cell wall during the same stages of its development in the same four trees.

The percentage composition of the carbohydrates in these components in the phloem is also reported.

EXPERIMENTAL

Materials used and general analytical methods

Pectic substances, α-cellulose and hemicellulose. These were prepared as described by Thornber & Northcote (1961).

Hydrolysis of polysaccharides. Each polysaccharide (5–10 mg.) was hydrolysed by the method of Saeman, Moore, Mitchell & Millett (1954). The neutralized hydrolysates were concentrated by distillation under reduced pressure at 50° on a water bath and made up to a volume of 1 ml. with water. The hydrolysates were stored in a deep-freeze at −16° until required.

Chromatographic separation of sugars in hydrolysates. Descending chromatograms were run on Whatman no. 1 papers with ethyl acetate–pyridine–water (8:2:1, by vol.) for 18 hr. for the separation of neutral sugars. For the separation of uronic acids descending chromatograms were run on Whatman no. 1 papers with ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) for 40 hr. A beaker containing ethyl acetate–pyridine–water (40:11:6, by vol.) was placed in the bottom of the chromatography tank (Fischer & Dörfel, 1955). E-MIL micropipettes were used to spot all quantitative chromatograms. Sugar spots on qualitative chromatograms were detected by spraying with aniline hydrogen phthalate (Partridge, 1949).

Quantitative estimations of sugars. After chromatographic separation, the individual neutral sugars were estimated with an accuracy of ±5%, by the method of Wilson (1959). The amount of uronic acid in each polysaccharide was estimated by the method of Dische (1947). When hexoses and pentoses are present in the samples being estimated, they contribute to the extinction at 530 mμ, and corrections were applied for the amount present in each sample. For 100 μg. of hexose 0·200 was subtracted from the extinction reading at 530 mμ, and for 100 μg. of pentose 0·012 was subtracted. A Unicam SP. 600 spectrophotometer was used for all extinction determinations.

Identification of 4-O-methylglucuronic acid in hydrolysates. Beech xylan, which is known to contain 4-*O*-methylglucuronic acid and xylose (Aspinall, 1959), was prepared from beechwood shavings by the method of Salkowski (1901). After hydrolysis and chromatography, the hydrolysate was found to contain two spots which reacted with aniline hydrogen phthalate. One spot moved with the same $R_{Glucose}$ as xylose; the other, 4-*O*-methylglucuronic acid, ran slightly faster ($R_{Glucose}$ 0·32) than glucuronic acid ($R_{Glucose}$ 0·27) in the system used for the separation of uronic acids (Fischer & Dörfel, 1955).

Identification of starch in hemicellulose preparations. Hemicellulose preparations were treated with a dilute solution of I_2 and examined under a microscope for a purple coloration.

Determination of the weight of a single cell in each region of each tree. These were determined as described by Thornber & Northcote (1961).

RESULTS AND DISCUSSION

Recovery of sugars after hydrolysis of polysaccharides and calculation of results. Known amounts of each monosaccharide were treated in exactly the same manner as the polysaccharides, and the percentage recovery of each sugar was determined after the hydrolysis procedure (Table 1). All results were expressed as the percentage of the total sugar estimated in each hydrolysate, after correction for losses and after expression as anhydro sugar units. In Tables 2–4 each anhydro sugar has been designated as a hexosan or pentosan; this is not meant to imply that they existed as distinct polysaccharides.

A chromatographic examination was also made of these hydrolysates to determine whether any epimerization of the original sugar had occurred during hydrolysis. Mannose was found in very small amounts in two of the twelve experiments which had used glucose as the starting material. The amount produced was so small that it could account for only a very small proportion of the mannose found in the hydrolysates of the carbohydrate fractions.

Uronic acids. Chromatography showed that the uronic acid present in the pectic substances was galacturonic acid only, and that 4-*O*-methylglucuronic acid with traces of galacturonic acid were present in all the hemicellulose preparations. In these fractions the total amount of uronic acid was estimated. The percentage of 4-*O*-methylglucuronic acid in the beech xylan was determined from the amount of methoxyl groups present (Pregl, 1937), and, when the uronic acid content of this material was determined by the method of Dische (1947), it was shown that a certain weight of 4-*O*-methylglucuronic acid gave the same extinction at 530 mμ as the same weight of galacturonic or glucuronic acid. Uronic acids were not detected in any α-cellulose preparations, either by chromatographic investigations or by examination of the absorption spectrum produced when these preparations were treated with sulphuric acid and carbazole.

Determination of the changes in the weight of each carbohydrate constituent of each main component of the cell wall during the differentiation of a cambial cell into the xylem. The comparison of the percentage composition of tissue in one region of a tree with that of tissues in another region will not give any indication of the changes in the structure of the

Table 1. *Percentage recovery of each monosaccharide after the hydrolysis procedure of Saeman* et al. *(1954)*

Values are given as percentage ±S.D. with number of experiments in parentheses.

Sugar	Percentage recovery
Galactose	62·6±3·8 (12)
Glucose	66·0±4·1 (12)
Mannose	69·0±3·3 (12)
Arabinose	63·2±3·6 (12)
Xylose	69·0±4·3 (12)
Rhamnose	66·0±4·0 (12)

tissue unless the differences in the weights of the cells between the two regions are measured (Thornber & Northcote, 1961). Recently some investigators (Meier & Wilkie, 1959; Sultze, 1957) have considered this change in cell weight whilst comparing the percentage composition of cells in different regions of a tree. Their results indicated that the cambial cell walls were rich in the pectic substances (araban, galactan and uronic anhydride components). In the aspen (*Populus tremuloides*) Sultze (1957) showed that large quantities of xylan were deposited during the laying down of the middle layer of the secondary cell wall, whereas in the pine (*Pinus sylvestris*) Meier & Wilkie (1959) found relatively large proportions of glucomannan deposited. During the later stages of the secondary cell-wall development large percentages of glucan and mannan in the aspen and xylan in the pine were laid down in the cell wall.

The results of these investigators were based on the assumptions such as this paper and the work of Thornber & Northcote (1961) have attempted to verify. Meier & Wilkie (1959) and Sultze (1957) determined the changes in the total carbohydrate composition of the cell wall; in our investigations we have fractionated the cell wall into the pectic substances, hemicellulose and α-cellulose, and determined the changes in the weight of each carbohydrate constituent of each main component as well as the changes in the carbohydrate composition of the whole cell wall during the differentiation of a cambial cell. The weight of each carbohydrate constituent of each main component was calculated from the weight of the main component (Thornber & Northcote, 1961) and its carbohydrate composition (Tables 2–4). The change in weight of the carbohydrate components of each main fraction during differentiation was determined by comparison of the composition of each fraction in the cambial, sapwood and heartwood region of each tree. The results are shown in Figs. 1–4.

General carbohydrate composition. The changes in the carbohydrate constituents of the whole cell wall are shown in Figs. 1–4. During secondary thickening of the cambial cell wall the glucans accounted for 56–67 % of the increase in weight of the carbohydrate components of the cell wall in the angiosperms, xylans 20–33 %, uronic anhydride 9–11 %, and mannans 2 % (birch and sycamore), 6 % (ash). There was very little change in the amount per cell of arabans (birch, −0·2 %; sycamore, 0·4 %; and ash, 0·6 %) and the amount of galactans decreased (−1 %). Comparison of these results with those for the pine in which the increase was made up by glucans 51 %, mannans 22 %, xylans 17 %, uronic anhydride 8 %, arabans 1·1 %, and galactans 0·3 % showed that a larger amount of the mannans was deposited during secondary thickening in this tree and that the amount of galactans increased. The increase in galactans and arabans may be due to the formation of galactoglucomannans and of arabogalactans which are known to occur in pine (Bishop, 1957; Hamilton, Partlow & Thompson, 1960).

Table 2. *Carbohydrate composition of the α-cellulose preparations from each tree*

Values are given as % (see text).

Anhydro sugar		Region of plug: Phloem		Cambium	Sapwood	Heartwood
Ash	Galactan	0·3		3·9	< 0·1	< 0·1
	Glucan	92·0		85·0	87·0	89·0
	Mannan	0·4		3·1	7·6	8·1
	Araban	1·8		4·6	0·0	0·0
	Xylan	5·7		3·8	5·2	3·3
Birch	Galactan	0·0		0·0	0·0	0·0
	Glucan	83·0		95·0	93·0	95·0
	Mannan	1·0		3·4	2·4	2·3
	Araban	6·0		0·6	0·1	0·0
	Xylan	10·3		1·5	4·0	2·7
		Outer	Inner			
Sycamore	Galactan	2·0	1·7	0·0	0·0	0·0
	Glucan	95·0	91·0	92·0	95·0	93·0
	Mannan	2·1	2·9	5·5	3·5	5·5
	Araban	0·2	1·6	0·6	0·1	0·1
	Xylan	2·8	2·8	1·9	1·2	1·5
Pine	Galactan	0·2		1·3	0·6	0·3
	Glucan	92·0		91·0	73·0	82·0
	Mannan	3·4		5·0	19·5	15·9
	Araban	2·5		1·3	1·1	0·2
	Xylan	1·9		1·7	5·1	2·3

Pectic substances. The pectic substances which were extracted from the cambial region of each tree with a solution of ethylenediaminetetra-acetic acid formed a higher percentage of the cell wall in this region than in any other region (Thornber & Northcote, 1961). The percentages of galactan and araban in the pectic substances (Table 4) from the cambium were higher than those found in most preparations of pectic substances (Northcote, 1958). Large percentages of these two constituents were also present in the hemicellulose prepared from cambial tissue. The percentage composition of the pectic substances from the cambial cell walls of the diffuse porous angiosperms was: galactan, 40–44%; araban, 15%; and polygalacturonic acid, 40–45%; these are expressed as a percentage of the total amount of these three constituents. For the ash the composition was: galactan, 26%; araban, 20%, and polygalacturonic acid, 54%; and for the pine: galactan, 28%; araban, 27%; and polygalacturonic acid, 45%.

The pectic substances contained glucans and xylans in addition to the sugars already mentioned. This may have been due to the inclusion in these preparations of small amounts of whole woody tissue. Courtois, Le Dizet & Davy (1960) have reported that pectic substances in the roots of *Silene inflata* contained similar high percentages of araban and galactan to those which we have found. They also reported the presence of rhamnose. We have found a small percentage of this sugar (0·1–6% of the weight of the preparation) in the pectic substances and in the hemicelluloses from each tree (Tables 3 and 4). The percentages of rhamnose were higher in the diffuse porous angiosperms than in the ash and pine. The percentage of hemicellulose in the cell wall in each region was greater than that of the pectic substances (Thornber & Northcote, 1961) and although a higher percentage of rhamnose occurred in the pectic substances, it accounted for only a small proportion of the total amount present. However, because of the relatively high percentage of rhamnose in the pectic substances, it would appear that it is definitely a constituent of this fraction. The cambial region of each tree contained a higher percentage of rhamnose than the phloem, which contained a larger percentage than the xylem. Rhamnose is thus de-

Table 3. *Carbohydrate composition of the hemicellulose preparations from each tree*

Values are given as % (see text).

		Region of plug			
	Anhydro sugar	Phloem	Cambium	Sapwood	Heartwood
Ash	Galactan	3·7	20·0	2·7	2·4
	Glucan	20·4	14·0	20·0	12·0
	Mannan	0·6	2·7	2·9	2·0
	Araban	4·9	5·3	2·5	2·1
	Xylan	54·0	49·0	56·0	59·0
	Rhamnan	Trace	0·5	Trace	Trace
	Uronic anhydride	16·2	10·0	16·0	23·0
Birch	Galactan	4·1	12·2	4·5	2·8
	Glucan	13·0	11·5	11·3	9·6
	Mannan	0·9	Trace	1·0	1·0
	Araban	6·8	3·9	1·6	1·8
	Xylan	62·0	53·0	63·0	65·0
	Rhamnan	0·2	0·5	0·2	Trace
	Uronic anhydride	12·5	19·5	19·0	19·0
		Outer / Inner			
Sycamore	Galactan	8·0 / 5·0	26·0	1·6	3·3
	Glucan	12·0 / 14·0	12·0	30·8	12·0
	Mannan	1·8 / 1·6	1·5	0·6	2·9
	Araban	3·8 / 3·3	4·2	1·0	3·3
	Xylan	53·0 / 49·0	43·0	47·0	57·0
	Rhamnan	2·5 / 2·9	3·0	0·3	0·3
	Uronic anhydride	21·0 / 28·0	13·0	19·0	21·0
Pine	Galactan	9·0	18·0	6·7	5·5
	Glucan	34·0	24·0	10·3	3·0
	Mannan	3·3	7·6	18·0	16·0
	Araban	11·5	13·0	5·7	6·0
	Xylan	27·0	25·0	38·0	44·0
	Rhamnan	Trace	0·2	Trace	Trace
	Uronic anhydride	16·0	12·0	21·0	20·5

posited in the cell wall during the formation of the primary cell wall rather than during secondary thickening. The presence of rhamnose has been reported in both hardwoods and softwoods (Hamilton & Thompson, 1959; Timell, Glaudemans & Gillham, 1959).

During secondary thickening, losses occurred in the araban and/or the galactan components of the pectic substances in each tree. The amount lost was very small compared with the increase in weight of the cell, but it represented 75% of the total amount of galactan and 63% of the araban in the pectic substances in the original cambial cell wall of each tree except the ash (Figs. 2–4). In the ash (Fig. 1) there was a very small increase in the weight of galactan in the pectic substances (6% of the amount in the cambial cell) and a loss in the weight of araban (23% of that in the cambial cell). During secondary thickening in all species a small deposition of polygalacturonic acid occurred, and, during the transition of a sapwood cell into the heartwood, there was a decrease in the amount of every component of the pectic substances in the sapwood cell.

The amount of galactans lost from each main component of the cell wall during differentiation was always greater than that for arabans; whereas if, as in some cases, an increase occurred in the amounts of these two components, that for arabans was always greater than that for galactans. Thus in the xylem (Figs. 1–4) and the phloem (Tables 2–4) the amounts of arabans to galactans was proportionally greater than in the cambial cell.

The almost complete absence of the pectic substances in the heartwood cell wall may be due to their being retained by the material deposited during secondary thickening and consequently not extractable with a solution of ethylenediaminetetra-acetic acid. If this were the explanation, it would be expected that the losses of the carbohydrate constituents of the pectic substances would be reflected as increases in the constituents of the other main components of the cell wall. This was not found in the angiosperms where the total carbohydrate composition showed a loss of galactan, and in some cases of araban as well, and thus it would appear that retention of the pectic substances did not occur and that the loss is a real removal of cell-wall constituents during growth. With the pine a similar conclusion could not be reached since in this tree the deposition of araboxylans (Garegg & Lindberg, 1960), galactoglucomannans and arabogalactans occurred during secondary thickening and thus the increase in the amount of arabinose and galactose in the tissue could not be directly related to the pectic material.

Table 4. *Carbohydrate composition of the pectic substances prepared from each tree*

Values are given as % (see text).

	Anhydro sugar	Region of plug: Phloem		Cambium	Sapwood	Heartwood
Ash	Galactan	6·0		24·0	17·0	6·0
	Glucan	5·5		7·5	19·0	28·0
	Araban	15·0		18·0	9·0	4·0
	Xylan	3·5		2·0	4·5	15·0
	Rhamnan	0·1		0·5	Trace	Trace
	Uronic anhydride	70·0		48·0	48·5	47·0
Birch	Galactan	7·0		38·5	7·0	6·0
	Glucan	21·0		9·0	26·0	39·0
	Araban	14·1		14·0	8·0	7·0
	Xylan	2·0		3·0	8·0	10·5
	Rhamnan	1·0		1·2	Trace	Trace
	Uronic anhydride	55·0		34·0	51·0	37·0
		Outer	Inner			
Sycamore	Galactan	8·5	4·0	35·5	5·0	4·8
	Glucan	28·0	5·0	8·1	15·3	15·7
	Araban	10·0	10·0	12·9	8·1	7·6
	Xylan	8·0	4·2	1·0	6·9	7·7
	Rhamnan	Trace	6·0	3·0	1·0	0·5
	Uronic anhydride	45·0	70·0	39·2	64·0	64·0
Pine	Galactan	7·0		23·0	7·0	6·2
	Glucan	11·1		13·5	13·2	12·9
	Araban	18·5		22·0	10·1	14·1
	Xylan	0·5		1·0	1·9	3·9
	Rhamnan	Trace		0·5	Trace	Trace
	Uronic anhydride	62·0		38·0	68·0	62·0

α-Cellulose. Most of the α-cellulose preparations (Table 2) contained less than 10% of their weight as non-glucan components. The exceptions were those from the xylem of the pine and from the phloem of the birch. In the former case, the lower percentage of glucan was due to the presence of a glucomannan (Meier, 1958) which can only be extracted when a mixture of alkali and boric acid is used (Jones & Painter, 1957). Larger percentages of mannans occurred in the α-cellulose in the xylem of the ash (8%) and pine (20%) than in any other region of the four trees. The composition of this fraction in the cambial region of each species was very similar, but in the other regions the carbohydrate composition differed in each species. Mannans represented 5% of the weight of the fraction in the cambial region, xylans (2%) and arabans (1%). During secondary thickening, the deposition of glucan accounted for 90% of the increase in weight of α-cellulose in the angiosperms (Figs. 1–3).

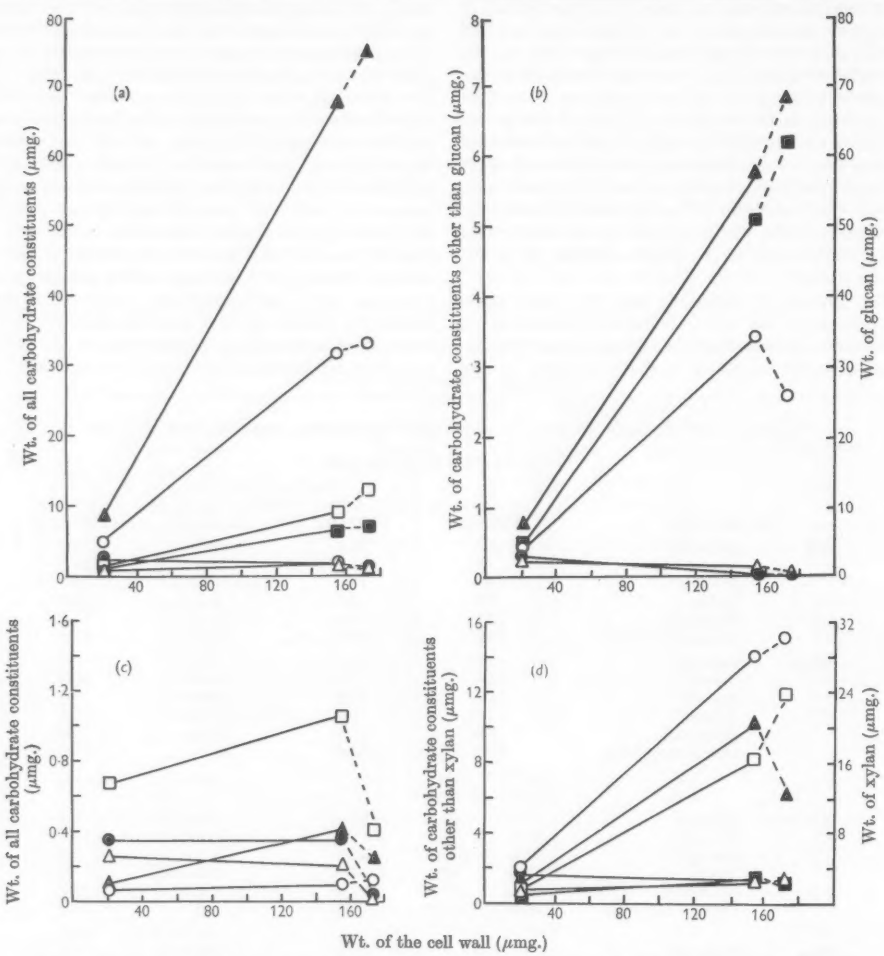
(a)
(b)
(c)
(d)
Wt. of all carbohydrate constituents (μmg.)
Wt. of carbohydrate constituents other than glucan (μmg.)
Wt. of glucan (μmg.)
Wt. of all carbohydrate constituents (μmg.)
Wt. of carbohydrate constituents other than xylan (μmg.)
Wt. of xylan (μmg.)
Wt. of the cell wall (μmg.)

Fig. 1. Weight of each carbohydrate constituent of each main component and the total carbohydrate composition at different stages of development in ash (*Fraxinus elatior*). (*a*), Total carbohydrate composition; (*b*), α-cellulose; (*c*), pectic substances; (*d*) hemicellulose. ●, Galactan; ▲, glucan; ■, mannan; △, araban; ○, xylan; □, uronic anhydride. ——, Cambial cell to sapwood development; - - - - -, sapwood cell to heartwood development.

The mannans and xylans accounted for the remaining 10%. In the gymnosperm, glucan accounted for 71% of the increase in weight and mannans 22% (Fig. 4). There was little alteration in the amount per cell of the arabans and galactans of the α-cellulose during differentiation. In the angiosperms there were small losses (−0·8%) and in the gymnosperm there was a small increase in their amounts (1·5%).

Hemicellulose. The hemicellulose preparations (Table 3) from the cambial region of each angiosperm had a very similar composition (galactans, 12–26%; glucans, 12–17%; mannans, 1–3%; arabans, 4–6%; xylans, 45–55%; and uronic anhydride, 15–20%), but the gymnosperm fraction contained a higher percentage of glucans, mannans and arabans with correspondingly lower percentages of the other constituents (galactans, 18%; glucans, 24%; mannans, 8%; arabans, 13%; xylans, 25%; and uronic anhydride, 12%). The increase in weight of the carbohydrates deposited in the hemicellulose of the xylem cell wall of the angiosperms during secondary thickening (Figs. 1–3) was made up mainly by xylans 47–66%; uronic anhydride, 18–20%, and glucans. The glucans showed the largest variation in the amount deposited in the different species and represented 33% of the material formed in the sycamore, 22%

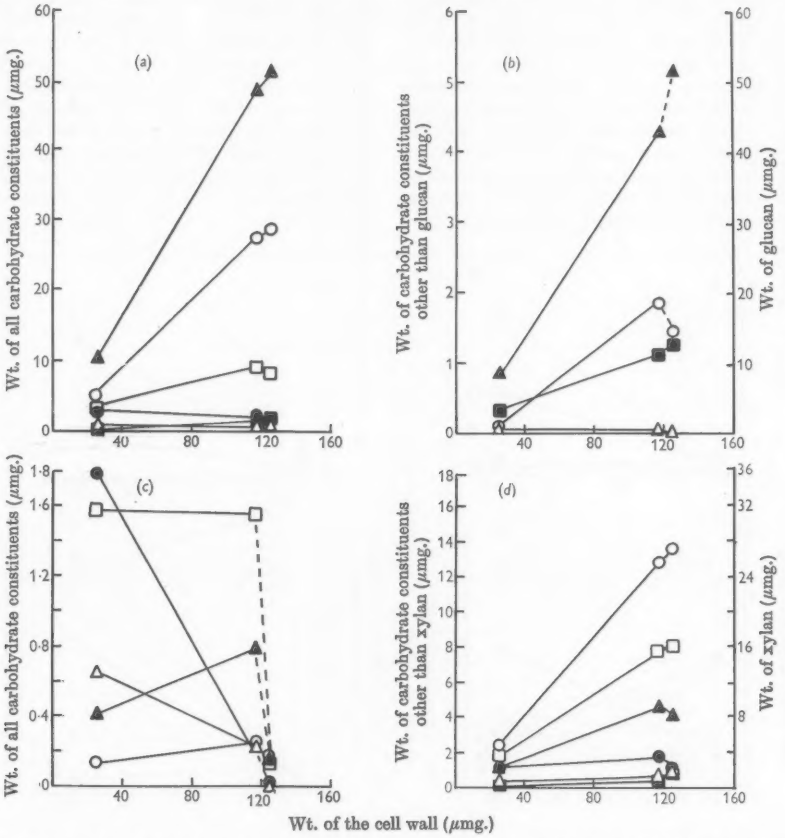
(a)
(b)
(c)
(d)
Wt. of all carbohydrate constituents (μmg.)
Wt. of carbohydrate constituents other than glucan (μmg.)
Wt. of glucan (μmg.)
Wt. of carbohydrate constituents other than xylan (μmg.)
Wt. of xylan (μmg.)
Wt. of the cell wall (μmg.)

Fig. 2. Weight of each carbohydrate constituent of each main component and the total carbohydrate composition at different stages of development in birch (*Betula platyphylla*). (*a*), Total carbohydrate composition; (*b*), α-cellulose; (*c*), pectic substances; (*d*), hemicellulose. ●, Galactan; ▲, glucan; ■, mannan; △, araban; ○, xylan; □, uronic anhydride. ——, Cambial cell to sapwood development; - - - - -, sapwood cell to heartwood development.

in the ash and 11% in the birch. The other constituents (arabans, galactans and mannans) represented less than 5% of the increase in weight. There was a loss of some of the galactans that were present in the hemicellulose of the primary wall in the ash and sycamore, whereas there was a small increase in the amount of arabans. A larger percentage of mannans (22%) and uronic anhydride (25%) and a smaller percentage of xylans (44%) and glucans (4·5%) were formed during secondary thickening in the pine. In this species galactans and arabans continued to be formed, but the amount formed was only 50% of the amount in the original cambial cell wall, whereas the cell wall had increased in weight by 410%.

The hemicellulose preparations from the phloem and cambium of the pine and that from the sapwood of the sycamore contained relatively high percentages of glucans (Table 3). All the hemicellulose preparations were tested for the presence of starch. It was not detected in the preparations from any cambial region or in those from the pine xylem. Very small amounts were present in the hemicellulose of the phloem of the pine and con-

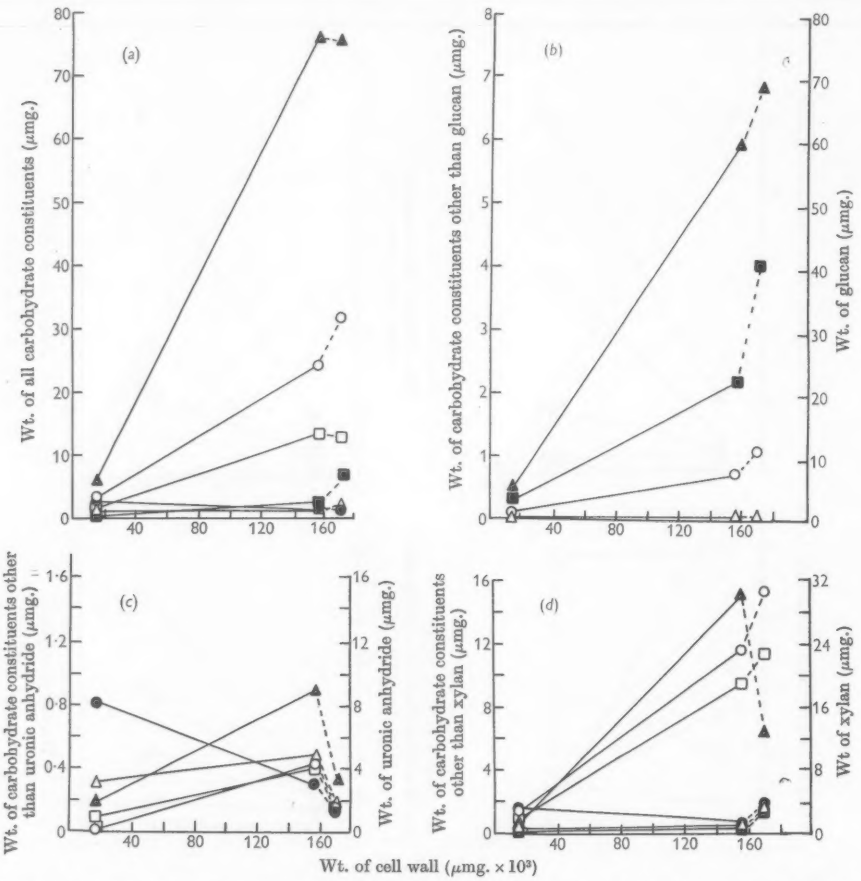
(a)
Wt. of all carbohydrate constituents (μmg.)
(b)
Wt. of carbohydrate constituents other than glucan (μmg.)
Wt. of glucan (μmg.)
(c)
Wt. of carbohydrate constituents other than uronic anhydride (μmg.)
Wt. of uronic anhydride (μmg.)
(d)
Wt. of carbohydrate constituents other than xylan (μmg.)
Wt of xylan (μmg.)
Wt. of cell wall (μmg. × 10³)

Fig. 3. Weight of each carbohydrate constituent of each main component and the total carbohydrate composition at different stages of development in sycamore (*Acer pseudoplatanus*). (*a*), Total carbohydrate composition; (*b*), α-cellulose; (*c*), pectic substances; (*d*) hemicellulose. ●, Galactan; ▲, glucan; ■, mannan; △, araban; ○, xylan; □, uronic anhydride. ——, Cambial cell to sapwood development; - - - - -, sapwood cell to heartwood development.

siderable amounts were present in the preparations from the other regions in the angiosperms. The origin of these glucan components is of interest, particularly as some preparations contained very little or no starch and yet appreciable quantities of glucans were present. The original material had been well extracted with water and little starch should have remained. Some of the glucans must have originated from glucomannans and this was known to be present in the hemicellulose and α-cellulose of some of the species of trees that we have studied. Glucomannans occur in pine wood (*Pinus sylvestris*) (Meier, 1958), in white birch (*Betula papifera*) (Timell, 1959) and in red maple (*Acer rubium*) (Mian & Timell, 1960). It is also possible that some of the glucans could have originated from a β-glucan, which may be present in the inner bark of white spruce (*Picea glauca*) (Painter & Purves, 1960) and which is known to occur as a food reserve in cereal grains (Aspinall, 1959). During the transition of a sapwood cell into the heartwood, the hemicellulose lost a certain amount of glucan, and Frey-Wyssling & Bosshard (1959) have found that the hydrolysis of starch seems to be a characteristic of this transition zone in ash (*Fraxinus elatior*) and pine (*Pinus sylvestris*).

Development. When the increases in the weights of each carbohydrate constituent of each main component are expressed as a percentage of the amount of that carbohydrate in the same component in the cambial cell, a very large percentage indicates that a constituent is formed during secondary thickening rather than during the formation of the primary cell wall. Calculated in this way our results showed that the mannans and xylans in the pine, the mannans in the ash and xylans in the birch were largely deposited during the secondary thickening of the cell wall. Similarly, it can be seen from the composition of the hemicellulose of each tree except the birch that, if all the uronic acid formed is 4-*O*-methylglucuronic acid (this was indicated by the chromatographic evidence), then either this uronic acid was being added to the xylan already deposited in the primary cell wall or the xylan polysaccharide formed during secondary thickening contained a higher percentage of 4-*O*-methylglucuronic acid than that formed during the development of the primary cell wall.

Synthesis of the xylan, uronic anhydride and, in some cases, the araban constituents of the hemicellulose continued during the transition of a sapwood cell into the heartwood in the angiosperms

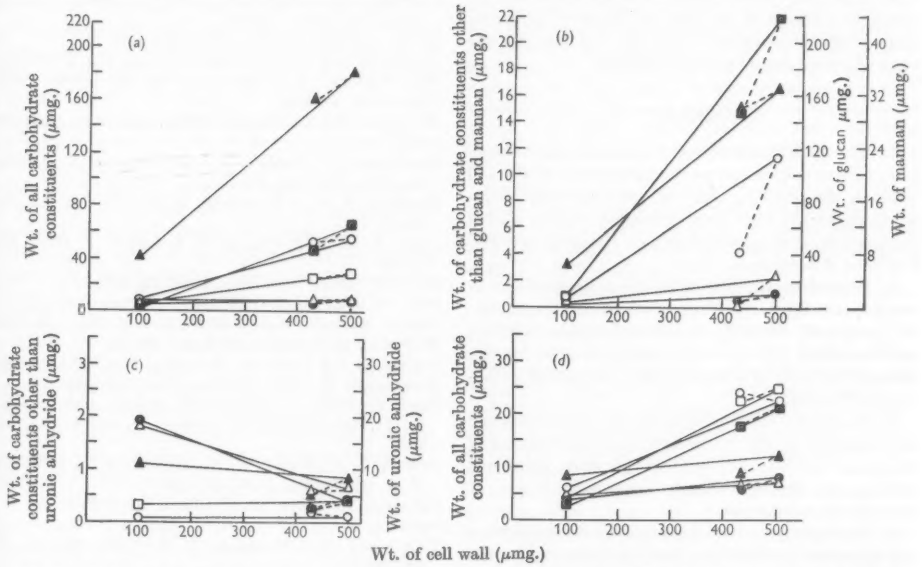
(a)
Wt. of all carbohydrate constituents (μmg.)
(b)
Wt. of carbohydrate constituents other than glucan and mannan (μmg.)
Wt. of glucan (μmg.)
Wt. of mannan (μmg.)
(c)
Wt. of carbohydrate constituents other than uronic anhydride (μmg.)
Wt. of uronic anhydride (μmg.)
(d)
Wt. of all carbohydrate constituents (μmg.)
Wt. of cell wall (μmg.)

Fig. 4. Weight of each carbohydrate constituent of each main component and the total carbohydrate composition at different stages of development in pine (*Pinus ponderosa*). (*a*), Total carbohydrate composition; (*b*), α-cellulose; (*c*), pectic substances; (*d*), hemicellulose. ●, Galactan; ▲, glucan; ■, mannan; △, araban; ○, xylan; □, uronic anhydride. ——, Cambial cell to sapwood development; - - - - -, sapwood cell to heartwood development.

(Figs. 1–3). The amount of hemicellulose formed during this transition is much smaller than that of α-cellulose (Thornber & Northcote, 1961), so that the additions to the α-cellulose, which were glucan and mannan only, would account for most of the material formed in the angiosperms. These results confirm those of Sultze (1957), who found that that part of the cell wall nearest the lumen of the cell in aspen contained large amounts of glucan and mannan. In the pine (Fig. 4), losses occurred in each carbohydrate constituent of each main component except that of xylan in the hemicellulose, and Meier & Wilkie (1959) have also found that in the pine the inner layer of the secondary cell wall contained a glucuronoaraboxylan.

Cambial tissue. The cell walls in the cambial region, although differing most noticeably from those in the other regions of the tree, are similar in each species both in the composition of the main constituents (Thornber & Northcote, 1961) and in their carbohydrate composition (Tables 2–4).

Phloem. The carbohydrate composition of the α-cellulose and hemicellulose components of the cell wall in the phloem (Tables 2–3) showed that they contained larger percentages of arabans and smaller percentages of mannans than in these components of the cell walls in other regions. The pectic substances in the phloem (Table 4) contained about the same percentage of arabans as the same preparations from the cambial cell walls, but the percentage of galactans was much smaller.

SUMMARY

1. The carbohydrate composition of the α-cellulose, hemicellulose and pectic substances of the cell walls in the phloem, cambial, sapwood and heartwood regions of four trees (*Fraxinus elatior*, *Betula platyphylla*, *Acer pseudoplatanus*, *Pinus ponderosa*) has been determined.

2. Secondary thickening in the angiosperms was accompanied by the formation of: glucans, 56–67%; xylans, 20–33%; uronic anhydride, 8–11%; and mannans, 2–6%. Little change occurred in the amount per cell of arabans. The amount of galactans decreased.

3. Secondary thickening in the gymnosperm was accompanied by the formation of larger amounts of mannans (22%), arabans (1%) and galactans (0·3%) and correspondingly lower percentages of the other constituents.

4. The xylans formed during secondary thickening appeared to contain greater amounts of uronic anhydride than that deposited in the primary cell wall.

5. The transition of a sapwood cell into the heartwood involved further formation of glucans and mannans in the angiosperms and of xylans in the gymnosperm.

6. Pectic substances (galactan, araban and polygalacturonic acid) are lost during the development of a cambial cell of the angiosperms into a heartwood cell.

7. Rhamnose appears to be a minor constituent of the pectic substances isolated from all four trees. It also occurs in the polysaccharides of the hemicellulose fraction and in both cases it is found only in the primary cell wall.

8. The carbohydrate composition of the cambial cell wall was very similar in each species.

9. The composition and development of the ash, a ring-porous angiosperm, were found to be intermediary between the diffuse porous angiosperm and the gymnosperm.

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Microbial Growth on C_1 Compounds

1. ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS* AM 1

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It has long been known that some non-photosynthetic micro-organisms can grow on organic C_1 compounds as their sole source of energy and carbon. The means whereby such organisms grow has been the subject of speculation. It has been suggested (Bhat & Barker, 1948; van Niel, 1954) that they might couple the energy of oxidation of the organic substrate to the reduction of carbon dioxide and the synthesis therefrom of all cell constituents. This would then be a variant of chemosynthetic autotrophy in which the organic compound would merely play a role similar to the inorganic electron donor. The occurrence of this autotrophic type of metabolism has been established by Quayle & Keech (1959*a*, *b*) in *Pseudomonas oxalaticus* grown on formate as sole carbon and energy source. In this organism, the cellular carbon is incorporated as carbon dioxide by the ribulose diphosphate cycle of carbon dioxide fixation (Bassham *et al.* 1954). More data are needed before the extent of occurrence of this cycle in general microbial growth on organic C_1 compounds can be assessed. It might be expected, especially with a C_1 substrate more reduced than formate, that the reduction level of the organic substrate might be conserved by at least some assimilation of carbon as reduced C_1 units, rather than synthesis of all cell material from carbon dioxide. Evidence has been obtained which indicates that such a heterotrophic type of metabolism operates during growth of *Pseudomonas methanica* on methane (Leadbetter & Foster, 1958) and *Pseudomonas* PRL–W4 on methanol (Kaneda & Roxburgh, 1959*a*, *b*, *c*). Few details of the process are known. For use in investigation of this problem, an organism has been isolated which grows rapidly on methylamine, methanol, formate, or a wide range of other compounds, in an otherwise mineral medium. Its isolation and characteristics are described in this paper.

A preliminary account of part of this work has been presented (Quayle & Peel, 1960).

METHODS

Maintenance and growth of the organism. All growth media contained the same concentrations of inorganic salts as those used by Jayasuriya (1955) for the growth of *Pseudomonas* OD1. For liquid culture the only addition was 0·5% (v/v) methanol (A.R.). Stock cultures were grown at 30°, on slopes of 0·1 M-methylamine hydrochloride with 1% (w/v) of agar-agar (Davis, New Zealand), stored at 2° and subcultured every 4 weeks. For growth on a large scale an inoculum was prepared by incubating 10 ml. of liquid medium for 24–48 hr. at 30° after sterile transfer of bacteria from the slope. The inoculum was introduced into a 1 l. conical culture flask (Fernbach flask), containing 200 ml. of liquid medium. The cells were then grown at 30° on a reciprocal shaker for 24–36 hr. and harvested by centrifuging whilst in the logarithmic growth phase at a cell density of 0·1–0·2 mg. dry wt./ml. Growth was determined by measurements with a nephelometer (Evans Electroselenium Ltd., Essex) previously calibrated in terms of dry wt. of cells/ml. of bacterial suspension.

Manometric methods. Consumption of O_2 was measured in conventional Warburg manometers at 30° with air as the gas phase. The cells were washed with, and resuspended in, aq. 0·9% KCl to a final concn. of about 4 mg. dry wt./ml.; 1 ml. of this cell suspension was placed in each main compartment of Warburg flasks, to each of which was added 100 μmoles of phosphate buffer (pH 7·2), prepared by adjusting 0·1 M-KH_2PO_4 to this pH by addition of 0·1 M-Na_2HPO_4. The volume of the contents of the main compartment was adjusted with water to give the flask contents a total volume of 2·2 ml. The centre well contained 0·2 ml. of 2 N-KOH. Sufficient substrate was added from the side arm to give a total theoretical O_2 uptake of 300–400 μl.

Electron microscopy. Dilute suspensions of the washed organism were fixed with 1% (w/v) OsO_4, placed on a Formvar-coated grid by the blot technique, and shadowed with gold–palladium (60:40). The photographs were taken in the Akashi Tronoscope, TRS 50.

Chemicals. A sample of β-hydroxy[^{14}C]butyric acid was a gift from Dr H. L. Kornberg.

Chromatographic analysis of pigments. The pigments were extracted from 1 g. of wet cells in two 10 ml. portions of chloroform–methanol (1:1, v/v). This solution was evaporated to dryness under N_2 at 60° and dissolved in 2 ml. of chloroform, ready for loading on a chromatography column. The column (250 mm. × 18 mm. internal diameter) was packed with 20 g. of silicic acid (Mallinckrodt Analytical Reagent, 100 mesh) slurried in chloroform. The silicic acid had been dried overnight at 120°. The column was developed with approx. 100 ml. portions of chloroform–methanol mixtures containing respectively 0, 1, 5 and 50% (v/v) of methanol. Fractions of 8–10 ml. were collected. During development the pigments separated into ten distinct bands all of which were red. The fractions containing these bands were evaporated to dryness under N_2 at 60°. The residues were dissolved in 4 ml. of absolute ethanol and their spectra measured over the range 250–650 mμ with a Cary recording spectrophotometer.

Identification of poly-β-hydroxybutyrate. Digestion of methanol-grown cells of organism AM 1 with alkaline hypochlorite at 37° (Williamson & Wilkinson, 1958) left a white, amorphous material which, after successive washing with water, acetone–ether (1:1, v/v) and ether, amounted to 6% of the dry weight of the organism. It was identified as poly-β-hydroxybutyrate on the basis of its melting point of 158–160°, and its hydrolysis to β-hydroxybutyric acid. This hydrolysis was performed in 2·5N-NaOH at 100° for 3 hr. After neutralization with HCl, samples of the resulting solution were chromatographed in the solvent systems described by Kornberg (1958): phenol–90% (w/v; A.R.) formic acid–water (500 g.: 13 ml.: 167 ml.) and by Benson *et al.* (1950): butanol–propionic acid–water (47:22:31, v/v). The acidic product, located by spraying with ethanolic bromocresol green (0·1%, w/v), co-chromatographed with authentic radioactive β-hydroxybutyric acid.

RESULTS

Isolation. During an investigation of bacterial growth on C_1 compounds as sole source of carbon and energy, heavy, pink growth, presumably due to some airborne contaminant, was observed in a medium containing 0·1M-methylamine hydrochloride and inorganic salts. This organism was isolated by repeated subculture in liquid medium and selection of single colonies from agar plates containing the same carbon source. The organism was designated AM 1.

Description. The organism is a Gram-negative rod measuring 0·8 μ × 2·0 μ and is motile with a single polar flagellum (Fig. 1). Colonies on agar are pink, raised, moist, circular and have smooth edges. They are pin-point within 2 days and reach a maximum diameter of 1 mm. after 1 week. The organism is strictly aerobic. Unshaken cultures produce a thin pellicle and shaken cultures have a tendency to clump which is particularly marked in growth on methylamine hydrochloride.

Growth characteristics

pH *Optimum.* This was determined in the medium of Jayasuriya (1955) except that the phosphate concentration was increased from 25 to 50 mM, and methanol was present as carbon source. The organism grows over the range pH 6·0–8·5 with a broad optimum at pH 7·0. No growth was observed at pH 5·0.

Temperature. The organism grows slowly at 25°, well at 30° and not at all at 37°.

Substrate concentration. Growth is reduced at methanol concentrations greater than 1% (v/v) and totally inhibited at 5% (v/v). Below these concentrations a mean generation time of 4–10 hr. is normally observed.

Growth on other substrates. Many compounds were tested as sole sources of carbon and energy at a concentration of 0·1% (w/v). The results of these experiments are shown in Table 1. Rapid growth to a cell density of about 0·2 mg. dry wt./ml. was observed on methanol, methylamine hydrochloride, sodium formate, ethanol, lactate, succinate, malate and fumarate. Slower growth was observed on glucose, fructose, aspartate, glutamate, glycollate and citrate. Negligible growth occurs on oxalate, glutarate, tartrate, alanine and mineral medium with no added carbon source. No trace of growth was found in the presence of formaldehyde (0·1 and 0·01%, w/v), glyoxylate, acetate, glycine, serine, propionate, glutarate and ribose. No growth was observed when the organism was incubated in liquid or on solid media without a carbon source other than that contained in an atmosphere of either methane (National Coal Board, Point of Ayr Unit, Flintshire, Wales)–air (1:1, v/v) or carbon dioxide–hydrogen–air (1:4:15, by vol.).

Growth factors. A requirement for added growth factors was not found since the organism grew normally after ten serial subcultures in liquid medium at a cumulative dilution of about 10^{-30}. The mean generation time of the organism was unchanged in the presence of 0·01 or 0·1% of yeast extract.

Pigment. The organism produced red intracellular

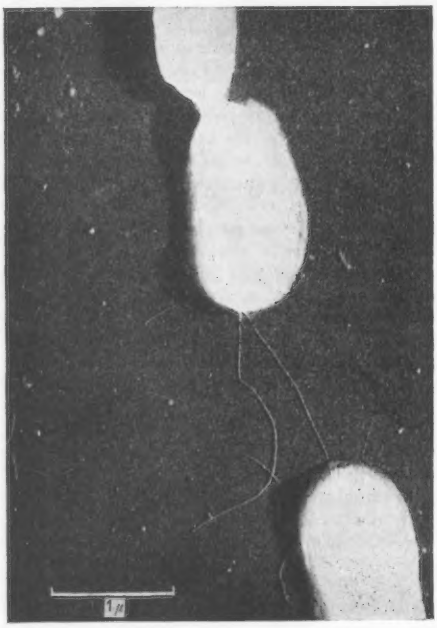

Fig. 1. Electron micrograph of *Pseudomonas* AM 1.

Table 1. *Comparison of the properties of organism AM 1 and other similar organisms*

++, Rapid growth; +, some growth; ±, difference between strains; −, no growth; ·, the property has not been reported.

Table 1. *Comparison of the properties of organism* AM 1 *and other similar organisms*

+ +, Rapid growth; +, some growth; ±, difference between strains; −, no growth; ., the property has not been reported.

	Pseudomonas methanica (*Methanomonas methanica*; Bergey)		*Pseudomonas* PRL–W4	*Pseudomonas aminovorans* (7 strains)	*Protaminobacter alboflavus* (4 strains)	*Protaminobacter ruber* (2 strains)	*Bacillus extorquens* (*Vibrio extorquens*)	Organism AM 1
References	Dworkin & Foster (1956), Leadbetter & Foster (1958), Harrington & Kallio (1960)		Kaneda & Roxburgh (1959*a*)		den Dooren de Jong (1926, 1927)		Bassalik (1913), Bhat & Barker (1948)	
Dimensions (μ) ...	0·4 × 1·5	0·3 × 0·7	0·5 × 1·2				Approx. 1·5 × 3·0	0·8 × 2·0
Flagella	Single polar	Single polar	Single polar	Single polar		Single polar*	Single polar	Single polar
Mean generation time (hr.)	.	.	72	.	.	.	.	4–10
Growth-factor requirements	−	−	Biotin	−	−	−	−	−
Pigmentation ...	Red, brown, yellow, colourless	Red	Red	Colourless or yellow	Colourless or yellow	Red	Red	Red
Utilization of carbon sources								
Methane	+ +	−	−	.	.	.	.	−
Methanol	+ +	+ +	+ +	±	−†	+†	+ +	+ +
Methylamine	.	.	.	+	−	+	.	+ +
Dimethylamine	.	.	.	±	−	+	.	+
Trimethylamine	.	.	.	+	−	+	.	+
Formaldehyde	−	−	−	.	.	.	+ +	−
Formamide	.	.	.	±	+	.	+	+
Formate	−	−	−	+	+	+†	+ +	+ +
Ethanol	−	+	−	±	+	−	+ +	+ +
Ethylamine	.	.	.	−	+	−	.	.
Diethylamine	.	.	.	±	±	±	.	.
Lactate	−	.	+ +	+	±	+	+	+ +
Succinate	−	.	+ +	+	+	+	+ +	+ +
Malate	−	.	−	+	+	+	+	+ +
Fumarate	−	.	+ +	+	+	+	+ +	+ +
Tartrate	−	.	+	±	±	±	+	−
Acetate	−	−	−	+	+	+	−	−
Glyoxylate	.	.	.	.	.	.	+ +	−
Glycollate	.	.	.	+	±	−	+ +	+
Oxalate	.	.	−	−	.	.	+ +	−
Propionate	.	.	−	+	.	.	−	−
Glutarate	.	.	+	±	±	+	+	−
Citrate	−	−	.	+	+	+	−	+
Glycine	−	.	−	−	.	.	+	−
Serine	.	.	.	.	.	.	.	−
Alanine	−	.	+ +	±	±	−	+	−
Aspartate	.	.	+ +	±	−	+	.	+
Glutamate	.	.	+ +	+	.	.	.	+ +
Glucose	−	.	+ +	+	±	+	+ +	+
Fructose	−	.	+	+	.	.	.	+
H_2–CO_2–air	−	.	.	.	.	.	.	−

* Weaver, Samuels & Sherago (1938).

† P. J. Large, unpublished observations with *Protaminobacter ruber* (N.C.I.B. No. 2879) and *P. alboflavus* (N.C.I.B. No. 8167).

pigments in the presence of all the carbon sources tested. The spectra of all of the fractions obtained by chromatography had absorption maxima at 526, 497, 472, 388, 315 and 270 mμ in ethanol, but the relative extinction coefficients at these maxima varied from one fraction to another. The spectrum of the main fraction is shown in Fig. 2. The spectra

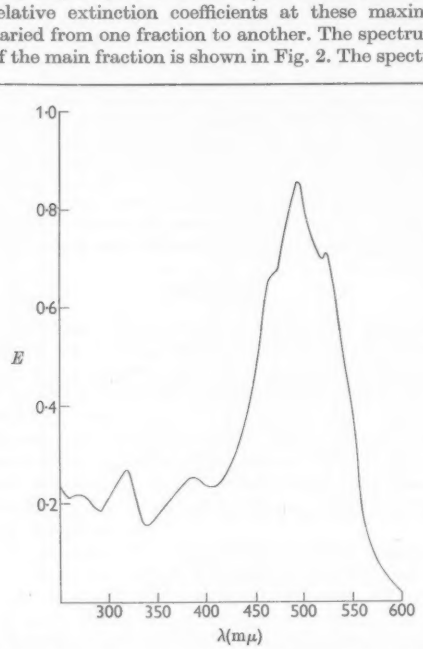

Fig. 2. Absorption spectrum of main pigment fraction from *Pseudomonas* AM1. The solvent was absolute ethanol.

and solubility of the pigments resemble those of highly unsaturated carotenoid pigments (Karrer & Jucker, 1950).

Presence of poly-β-hydroxybutyrate. Methanol-grown cells of organism AM1 contained 6% of their dry weight in the form of a white, amorphous material which was identified as poly-β-hydroxybutyrate on the basis of its melting point and alkaline hydrolysis to β-hydroxybutyric acid.

Oxidative properties. The oxidation of various substrates by washed, whole cells of the organism grown on methanol, formate, or succinate is recorded in Table 2. When grown on any of these substrates, the organism is able to oxidize both formate and formaldehyde. With all substrates tested, the total oxygen uptake was 50–70% of the theoretical value for complete combustion. An increase of about 10% in the total oxygen uptake with methanol, formaldehyde and formate was observed in the presence of 1 mM-2:4-dinitrophenol. The oxidation of formate was partially inhibited by 1 mM-sodium hypophosphite, a compound which is an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953). Tris hydrochloride was not oxidized by the cells, nor did its presence alter the rate of oxidation of methanol.

DISCUSSION

The properties of organism AM1 lead us to classify it as a species of *Pseudomonas* (Bergey, 1957), and we propose to refer to it as *Pseudomonas* AM1. A culture so named has been deposited with the National Collection of Industrial Bacteria, Torry, Aberdeen.

Table 2. *Oxidative properties of washed suspensions of organism* AM1 *grown on methanol, formate and succinate*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (4 mg. dry wt.); 100 μmoles of Na_2HPO_4–KH_2PO_4 buffer, pH 7·2; side arm, substrate; centre well, 400 μmoles of KOH. Total volume of contents was 2·2 ml. Incubation was at 30° for a maximum of 3–4 hr. (For further details see Methods.)

	Growth substrate: Methanol		Formate		Succinate	
Substrate for oxidation	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}
Methanol	−187	−13	−146	−8	−32	−29
Formaldehyde	−199		−121		−65	
Formate	− 99		− 53		−39	
Methylamine	−163		− 57*			
Succinate	− 43		− 52	−19	−73	
Malate	− 48		− 55		−81	
Fumarate	− 45		− 37			
Citrate	− 13		− 21			
Glycine	− 13		− 41			
Serine	− 13		− 32			

* After a lag of 3 hr.

The organisms listed in Table 1 are similar to *Pseudomonas* AM 1 in various respects. They are all Gram-negative rods which can grow aerobically on C_1 compounds as sole sources of carbon and energy. Most of them are motile with a single polar flagellum and some of them contain carotenoid-like pigments. With the exception of *Pseudomonas methanica* (*Methanomonas methanica*; Bergey, 1957), they will also grow aerobically on a wide range of organic acids, simple amines or glucose as sole sources of carbon and energy. *P. methanica*, like *Pseudomonas* AM 1, has been found to contain poly-β-hydroxybutyrate (Kallio & Harrington, 1960). In view of the important similarities between these organisms, their classification into different genera may not in fact be justified. Indeed, Dworkin & Foster (1956) and Slepecky & Doetsch (1954) have questioned the establishment of the genera *Methanomonas* and *Protaminobacter*, and suggest that organisms so named could equally well be classified as *Pseudomonas*.

The relative ease with which *Pseudomonas* AM 1 grows on several C_1 compounds in a defined medium makes it a valuable organism for study of microbial growth on such compounds, and the following paper describes initial studies on the metabolism of methanol and formate by cells grown on these substrates.

SUMMARY

1. An organism has been isolated which grows aerobically on methylamine, methanol, formate or formamide as sole sources of carbon and energy. Neither methane nor formaldehyde serves as growth substrate, nor can the organism grow autotrophically at the expense of oxidation of hydrogen. Certain mono- and di-carboxylic acids, amino acids, amines and glucose will also support growth in an otherwise mineral medium.

2. The organism is a pink-coloured, Gram-negative, motile rod ($0{\cdot}8\,\mu \times 2{\cdot}0\,\mu$) with a single polar flagellum. It is identified as a species of *Pseudomonas* and is referred to as *Pseudomonas* AM 1.

3. With methanol as growth substrate, the optimum growth conditions of *Pseudomonas* AM 1 are obtained at pH 7, at a temperature of 30°, and at a methanol concentration of 0·5–1 % (v/v). No requirement for any growth factor was found.

4. The oxidative abilities of the methanol-, formate- and succinate-grown organism have been examined manometrically.

5. The solubility and spectral characteristics of the pink pigments suggest that they are unsaturated carotenoids.

6. Methanol-grown *Pseudomonas* AM 1 contains poly-β-hydroxybutyrate to the extent of about 6 % of its dry weight.

7. The properties of *Pseudomonas* AM 1 are compared with those of other similar organisms.

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Microbial Growth on C_1 Compounds

2. SYNTHESIS OF CELL CONSTITUENTS BY METHANOL- AND FORMATE-GROWN *PSEUDOMONAS* AM 1, AND METHANOL-GROWN *HYPHOMICROBIUM VULGARE*

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The preceding paper (Peel & Quayle, 1961) described the isolation of an organism, *Pseudomonas* AM 1, which can grow on methanol or formate as sole sources of carbon and energy. The present paper describes an investigation of the path of carbon incorporation from [^{14}C]methanol, [^{14}C]-formate, and [^{14}C]bicarbonate into cell constituents during growth of this organism on methanol or formate. Similar experiments, involving [^{14}C]methanol and [^{14}C]bicarbonate, have been performed with methanol-grown *Hyphomicrobium vulgare*, and the data obtained with the two organisms are compared.

METHODS

Maintenance and growth of the organisms. The maintenance and growth of cultures of *Pseudomonas* AM 1 have been described (Peel & Quayle, 1961).

A culture of *H. vulgare* was a gift from Dr R. Näveke (Staatsinstitut für Allgemeine Botanik, Jungiustrasse 6, Hamburg 36, Germany). This organism was maintained on agar slopes, containing 0·1 M-methylamine hydrochloride as carbon source, and grown in liquid culture on methanol (0·5%, v/v), in similar media to those used for *Pseudomonas* AM 1. With the liquid culture, the growth flasks were not shaken, and the organism was harvested after about 2 weeks. *H. vulgare* grew very slowly as a slime on the internal glass surface of the culture flask, and was harvested by scraping it off the glass and centrifuging the resulting suspensions.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. [^{14}C]Methanol was supplied in a sealed tube under vacuum. One end of the tube was cooled in liquid air and the other end was then opened. Water was added to the methanol whilst the tube was still in liquid air. After slow thawing, the solution of [^{14}C]methanol was transferred to a stoppered tube and stored at $-10°$.

$Ba^{14}CO_3$ was decomposed with an excess of 2 N-$HClO_4$ and the liberated $^{14}CO_2$ was trapped in the theoretical quantity of NaOH by using a transfer unit similar to that described by Aronoff (1956).

Sodium [^{14}C]formate was chromatographed on Whatman no. 4 filter paper with a solvent system (Long, Quayle & Stedman, 1951) consisting of ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (80:4:16, v/v). After development, the papers were sprayed with 0·5 M-K_2HPO_4 and dried in air, and the formate area was located by radioautography. The radioactive area was eluted with water into one arm of a transfer unit (Aronoff, 1956) and the formic acid was liberated with 5 N-H_2SO_4 and distilled *in vacuo* into the theoretical quantity of NaOH solution.

Measurement of specific activity of radioactive bacterial cells. The specific activity of cells after growth on radioactive substrates was measured by two different methods, outlined below:

(*a*) Direct plating of bacterial cells. Samples of the growing culture were withdrawn at intervals, their cell density was measured with a nephelometer (Evans Electroselenium Ltd., Halstead, Essex), and the suspensions of cells were centrifuged at 0°. The supernatant solutions were poured off and each sample of the cells was resuspended in 0·5 ml. of water; samples (0·1–0·2 ml. containing $< 0·6$ mg. dry wt.) of the resuspended cells were plated on glass planchets (3 cm.^{2} area) on a rotating turntable in a stream of warm air. Each planchet was washed with 0·1 ml. of formic acid (90% w/v; A.R.) and dried, and the radioactivity assayed, unless stated otherwise, with a gas-flow counter (Model D–47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.). A blank determination showed that a negligible amount of [^{14}C]methanol remained on the planchets after this treatment.

(*b*) Combustion of bacterial cells. The cells were washed three times with aq. 0·9% KCl to remove exogenous radioactive compounds and were then totally oxidized to CO_2 in the combustion apparatus described by Sakami (1955*a*). The evolved CO_2, collected as $Na_2{}^{14}CO_3$ in 2·5 N-NaOH, was converted into $Ba^{14}CO_3$ by precipitation with aq. 5% (w/v) $BaCl_2$. The $Ba^{14}CO_3$ was plated on paper disks (2·69 cm.^{2} area) as described by Sakami (1955*b*), assayed for radioactivity in the gas-flow counter, and corrected for self-absorption.

Bacterial incorporation of [^{14}C]*methanol*, [^{14}C]*formate and* [^{14}C]*bicarbonate.* Cultures of bacteria in the logarithmic growth phase were centrifuged at room temperature and resuspended in solutions containing the growth substrate, buffer and, in some cases, other salts (see text) to give a concentration of about 3 mg. dry wt. of bacteria/ml. With *H. vulgare* it was not practicable to ascertain if the cells were in the logarithmic phase of growth when harvested, nor could the cell density be estimated nephelometrically. Instead, the harvested organism was pressed between filter paper to remove excess of water and then weighed out directly into the incubation mixtures to give a final concentration corresponding to about 3 mg. dry wt./ml.

Portions (9–10 ml.) of these bacterial suspensions were placed at 30° in cylindrical vessels (10 cm. × 2·5 cm. diam.) fitted with sintered-glass bottoms and vigorously aerated by blowing a stream of moist air through the sintered glass. To each suspension was added 200 μc of ^{14}C, and 1 ml. samples were withdrawn with a hypodermic syringe at suitable time intervals over 5 min. All samples were

transferred immediately to 3 ml. of boiling aq. 95% (v/v) ethanol. The ethanolic suspensions of killed cells were centrifuged and the resulting precipitates extracted with 0·5 ml. of boiling aq. 20% (v/v) ethanol. The combined supernatants were evaporated to dryness at 60° under N_2, taken up in 0·1 ml. of aq. 20% (v/v) ethanol and applied to the origin position of paper chromatograms.

Chromatographic analysis and radioautography. The papers (Whatman no. 4; 18¼ in. × 22 in.) were developed two-dimensionally in a descending direction, by using first the solvent system of Kornberg (1958): phenol–90% (w/v, A.R.) formic acid–water (500 g.: 13 ml.: 167 ml.). The solvent system *n*-butan-1-ol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950) was used to develop the chromatogram in the second dimension. Both solvents were allowed to run to the edge of the paper.

Radioautographs were made from the chromatograms by the procedure developed by Calvin and his co-workers (see Calvin, 1950–51), with Blue Brand X-ray film (Kodak Ltd.). The radioactive areas located on the paper were assayed for radioactivity directly with a mica end-window Geiger–Müller tube (General Electric Co.; type 2B2) and the count was corrected for background activity.

Under the conditions of chromatography used, glutamic acid, serine and glycine did not usually separate from each other. The radioactive area containing them was cut out, eluted with water, and the amino acids were separated by further chromatography or electrophoresis (see below). The proportion of each amino acid in the radioactive area on the parent chromatogram was assumed to be that found after separation, even though losses of total count occurred during the overall procedure.

Identification of labelled compounds. The radioactive compounds were eluted from the chromatograms with water and subjected to co-chromatography or co-electrophoresis with authentic samples of the suspected compound. Exact matching of the resulting radioactive area and the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were re-chromatographed either in the original solvent systems or ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (80:4:16, by vol.; Long *et al.* 1951), or subjected to high-voltage electrophoresis at 100 v/cm. in 0·5 M-acetic acid adjusted to pH 4·0 with pyridine (Gross, 1959). Amino acids were either re-chromatographed in butan-1-ol–acetone–water–diethylamine (40:40:20:8, by vol.; Hardy, Holland & Nayler, 1955) or subjected to high-voltage electrophoresis at 100 v/cm. in 0·75 M-formic acid (pH 2·0; Gross, 1959). Both these methods separated glutamic acid, glycine and serine from each other; the electrophoretic system also separated aspartic acid from glutamic acid. Dephosphorylation of phosphorylated compounds was carried out by incubation of the eluted compounds with Polidase-S (Schwarz Laboratories, Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951), and the resulting compounds were chromatographed two-dimensionally in water-saturated phenol and butanol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950).

Trehalose was identified as follows: (*a*) by co-electrophoresis with authentic trehalose in 0·05 M-sodium borate at 100 v/cm. for 30 min. (Cabib & Leloir, 1958); (*b*) by its partial hydrolysis in N-HCl at 100° for 30 min., which yielded glucose as the sole product; (*c*) by admixture with authentic trehalose, after which the specific radioactivity of the resulting material remained constant over two successive recrystallizations from aq. 80% (v/v) ethanol.

Sprays. Carboxylic acids were located on chromatograms by spraying with a solution (0·2%, w/v) of bromocresol green in ethanol and adjusting to pH 7 with aq. NH_3 solution; amino acids by spraying with 0·2% (w/v) ninhydrin in ethanol containing 0·1% (v/v) of collidine, followed by heating at 100°; aldose sugars by spraying with a solution of aniline (0·9 ml.) and phthalic acid (1·7 g.) in 100 ml. of water-saturated butanol, followed by heating at 105° (Partridge, 1949); fructose by spraying with 1% (w/v) naphtharesorcinol in ethanol–2 N-HCl (10:90, v/v; Forsyth, 1948) followed by heating to 90° for 10 min.; sedoheptulose by spraying with a solution of orcinol (0·5 g.) and trichloroacetic acid (15 g.) in 100 ml. of water-saturated butanol, followed by heating at 100° (Klevstrand & Nordal, 1950); trehalose by spraying first with $AgNO_3$ in aq. acetone, followed by 0·5 N-NaOH in aq. ethanol (Trevelyan, Procter & Harrison, 1950).

Preparation of cell-free extracts. Cell-free extracts of methanol-grown *Pseudomonas* AM1 were prepared either by crushing frozen bacteria in a Hughes (1951) press, or by ultrasonic disintegration. For formate-grown *Pseudomonas* AM1 and methanol-grown *H. vulgare*, the method of ultrasonic disintegration was not used. For crushing the cells, 1 g. wet wt. of bacteria was suspended in 2 ml. of 30 mM-tris buffer, pH 7·5, containing about 200 μg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.). The suspension was crushed in a Hughes press which had been cooled to −25°. For preparing sonic extracts, 1 g. wet wt. of cells was suspended in 5 ml. of solution containing 100 m-moles of cysteine and 150 m-moles of tris buffer, pH 7·8. This suspension was cooled to 0° and subjected for 2 min. to the output at 25 kcyc./sec. of a 600 w Mullard magnetostrictor oscillator. The extracts resulting from both processes of cell breakage were either used as such, or after centrifuging at 25000*g* for 10 min. in a refrigerated centrifuge.

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

Chemicals. Ribulose 1:5-diphosphate was prepared by incubating ribose 5-phosphate and ATP with purified phosphoriboisomerase and phosphoribulokinase prepared from spinach according to the method of Horecker, Hurwitz & Weissbach (1956).

RESULTS

Effect of flushing Pseudomonas AM1 *with carbon dioxide-enriched air while growing on* ^{14}C*-labelled substrates*

In order to estimate the amount of cellular carbon derived from the growth substrate which had passed through a stage readily exchangeable with carbon dioxide during growth, two cultures of the organism were grown on each ^{14}C-labelled substrate. The $^{14}CO_2$ derived from oxidation of the substrate was removed from one culture of each pair by passing a stream of air–carbon dioxide (99:1, v/v) through the flask. The remaining flask of each pair was flushed with carbon dioxide-free air. The specific radioactivity of the cells in each culture was then measured and compared.

Attempts to perform this type of experiment with formate-grown *Pseudomonas* AM1 were not successful, owing to difficulties experienced in growing the cells in a stream of carbon dioxide-free air. The experiment was not attempted with *H. vulgare*, owing to its very slow rate of growth.

Growth on [^{14}C]*methanol.* Growth medium (400 ml.) containing [^{14}C]methanol (100 m-moles; 75 μc of ^{14}C) as carbon source was inoculated with *Pseudomonas* AM1. The suspension was divided equally between two conical culture flasks, one of which was flushed with carbon dioxide-free air and the other with carbon dioxide–air (1:99, v/v). The flasks were shaken at 30° and the bacteria were grown through about four generations, as determined nephelometrically. Samples were withdrawn at intervals, and the specific radioactivity of the cells was determined by direct plating on glass planchets followed by assay of radioactivity with a lead-shielded, end-window Geiger–Müller tube. The specific activity of the cell material rose to a constant value in each case, and remained constant during two generations of growth (Fig. 1). The cells grown in air reached an activity of 5·4 counts/min./μg. dry wt. of cell material. Those grown in air–carbon dioxide reached an activity of 2·7 counts/min./μg. dry wt. The experiment was repeated, with [^{14}C]methanol of higher specific activity (50 m-moles; 500 μc of ^{14}C), and this time the specific activity of the cells was measured as $Ba^{14}CO_3$ derived from the cells by total combustion. The specific activity of the cells grown in air was found to be 6090 counts/min./mg. of $Ba^{14}CO_3$, a figure reduced to 3330 counts/min./mg. of $Ba^{14}CO_3$ in the cells grown in air–carbon dioxide.

The results from these experiments, which are in close agreement, indicate that at least 50 % of the carbon entering the cells from methanol passed through a stage exchangeable with exogenous carbon dioxide.

Growth on [^{14}C]*succinate.* The previous experiment was repeated with cultures of *Pseudomonas* AM1 growing on uniformly labelled [^{14}C]succinate. The specific radioactivity of the cells was measured by total combustion to $^{14}CO_2$, which was assayed as $Ba^{14}CO_3$. Flushing the cells with air–carbon dioxide (99:1), instead of air, reduced the specific activity of the cells from 375 to 349 counts/min./mg. of $Ba^{14}CO_3$, i.e. a reduction of 7 %.

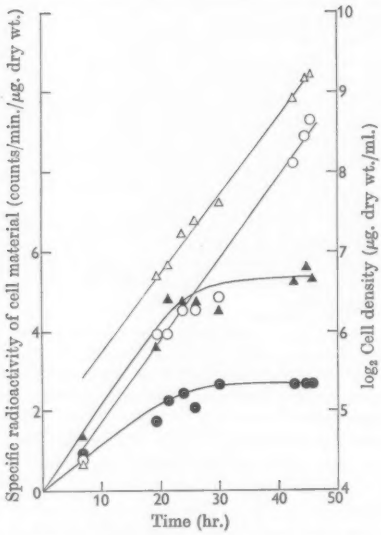

Fig. 1. Effect of flushing *Pseudomonas* AM1 with CO_2-enriched air during growth on [^{14}C]methanol. The specific activity of the cellular material was determined by direct plating on glass planchets. The experimental conditions are described in the text. △, ▲, Growth and specific radioactivity respectively of cells grown under CO_2-free air; ○, ●, growth and specific radioactivity respectively of cells grown under air–CO_2 (99:1).

Analysis of path of labelled substrates into cell constituents

Incorporation of [^{14}C]*methanol and* [^{14}C]*bicarbonate by methanol-grown* Pseudomonas AM1. A culture of bacteria in the logarithmic growth phase was centrifuged and resuspended in a solution of 20 mM-methanol in 10 mM-tris buffer (pH 7·8) to give a concentration of 3 mg. dry wt. of bacteria/ml. Two 10 ml. portions of this suspension were then used for incubation with [^{14}C]methanol and [^{14}C]bicarbonate respectively. Chromatographic analysis, followed by radioautography, showed that radioactivity from either tracer rapidly appeared in a large number of compounds. The distribution of radioactivity among the labelled compounds formed during the first 2 min. after the addition of isotope is shown in Tables 1 and 2. The curves obtained by plotting against time the percentage of the total radioactivity fixed which was contributed by each constituent of the samples are shown in Figs. 2 and 3. With [^{14}C]methanol the only curves clearly showing a negative slope immediately after the introduction of isotope are those of aspartate and serine, the latter containing the majority of the radioactivity in the first sample. The initial slope of the glycine curve may also be negative, although the very small amount of radioactivity appearing in this compound does not permit an accurate evaluation of this. Other compounds such as malate, phosphorylated compounds, citrate, glutamate and the disaccharide, trehalose, acquired initially increasing percentages of the total radioactivity fixed. At the end of 2 min., trehalose

contained the highest percentage of radioactivity. Chromatographic analysis of the compounds resulting from dephosphorylation of the phosphate area of the chromatogram showed a mixture of phosphates of glyceric acid, glucose, fructose, ribose and sedoheptulose to be present. For incubation of [^{14}C]bicarbonate with the organism, most of the count at early times was contained in malate. From 10 sec. onwards the malate curve showed a steep negative slope; whether the apparent rise in the curve between 4 and 10 sec. is of significance or is due to the errors attendant on the low fixation obtained in the earliest sample is not known at present. Both glycine and serine showed initially negative slopes, although the total count in either compound was relatively small. As with [^{14}C]methanol, radioactivity rapidly accumulated in trehalose at later times.

Incorporation of [^{14}C]*formate and* [^{14}C]*bicarbonate by formate-grown* Pseudomonas AM 1. Similar experiments to those described above were performed on [^{14}C]formate and [^{14}C]bicarbonate incorporation by formate-grown *Pseudomonas* AM 1. The distribution of radioactivity among the labelled compounds formed is shown in Tables 3 and 4, and the curves of percentage of total radioactivity fixed which was contributed by each constituent of the sample, plotted against time, are shown in Figs. 4 and 5. With [^{14}C]formate as tracer, the curves belonging to serine and malate show pronounced negative slopes initially, whereas the curves relating to phosphorylated compounds and, later, trehalose, show positive slopes (Fig. 4). The percentage of radioactivity contained in glycine remained approximately constant. When [^{14}C]bicarbonate was used (Fig. 5), only the curve relating to

Table 1. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown* Pseudomonas AM 1 *incubated with* [^{14}C]*methanol*

[^{14}C]Methanol (38 μmoles; 200 μc of ^{14}C) was added at zero time to a 10 ml. suspension of bacteria (30 mg. dry wt.) in 20 mM-methanol, 10 mM-tris buffer (pH 7·8), under vigorous aeration. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and transferred at known times into 3 ml. of hot ethanol. The precipitates were extracted with 1 ml. of aq. 20% (v/v) ethanol and the combined extracts analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window Geiger–Müller tube.

	Radioactivity (counts/min.)									
Time (sec.)	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds	Other compounds	Total
4	9	0	18	0	6	0	0	5	9	47
9	22	0	23	0	6	0	0	9	6	66
14	52	0	37	0	10	0	0	29	11	139
20	100	11	38	0	11	7	0	56	35	258
24	101	10	41	12	12	8	12	81	44	321
30	133	9	71	0	16	14	84	125	72	534
46	90	17	48	11	18	31	75	188	91	569
60	57	17	47	30	22	56	137	191	154	711
122	167	50	60	200	19	74	614	372	599	2155

Table 2. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown* Pseudomonas AM 1 *incubated with sodium* [^{14}C]*bicarbonate*

Sodium [^{14}C]bicarbonate (100 μmoles; 200 μc of ^{14}C) was added at zero time to a 10 ml. suspension of bacteria (30 mg. dry wt.) under conditions identical with those described in Table 1.

	Radioactivity (counts/min.)									
Time (sec.)	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds	Other compounds	Total
4	62	23	10	0	17	20	0	2	44	178
10	134	28	19	9	30	28	0	6	61	315
15	283	43	40	18	51	67	0	45	274	821
21	273	55	28	19	42	118	0	49	320	904
26	184	41	24	18	37	81	16	54	290	745
32	189	70	31	47	42	142	33	121	435	1110
55	127	46	35	132	62	302	198	185	471	1558
64	122	49	18	137	63	343	254	154	351	1491
121	358	109	59	494	63	617	1066	271	884	3921

malate showed an initially negative slope. Radioactive glycine was present at the earliest times, containing approximately 7% of the total radioactivity fixed.

Incorporation of [^{14}C]*methanol and* [^{14}C]*bicarbonate by methanol-grown* Hyphomicrobium vulgare. Similar experiments to those described above were performed on [^{14}C]methanol and [^{14}C]bicarbonate incorporation by methanol-grown *H. vulgare*. The distribution of radioactivity amongst the labelled compounds formed is shown in Tables 5 and 6, and the curves of percentage of total radioactivity fixed which is contributed by each constituent of the sample, plotted against time, are shown in Figs. 6 and 7.

With the [^{14}C]methanol incorporation, radioactive serine and glycollate contained most of the initially incorporated tracer, and the curves relating to both these compounds showed clear negative slopes throughout the experiment (Fig. 6). All other compounds showed initially positive slopes. The percentage of radioactivity contained in glycine fluctuated between 3 and 6% of the total. No trehalose was detected. When [^{14}C]bicarbonate was the isotope used (Fig. 7), the curves relating to malate and aspartate showed initially negative slopes; curves of other compounds (including glycine) showed positive slopes. No radioactive glycollate was detected.

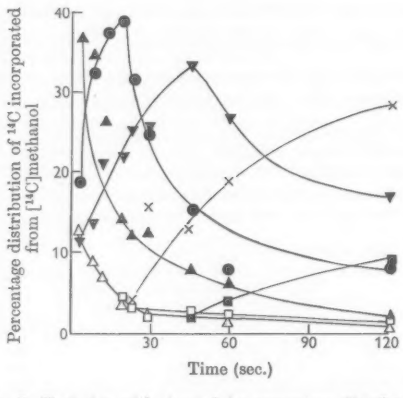

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]methanol into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1, growing on methanol. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

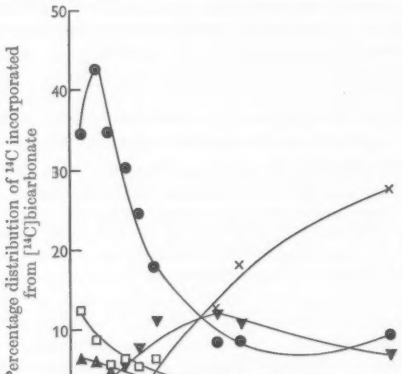

Fig. 3. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]bicarbonate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on methanol. ●, Malate; ▲, serine; □, glycine; ▼, phosphorylated compounds; ×, trehalose.

Table 3. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of formate-grown* Pseudomonas AM1 *incubated with* [^{14}C]*formate*

Sodium [^{14}C]formate (32·7 μmoles; 200 μc of ^{14}C) was added at zero time to 8 ml. of a suspension of bacteria (31 mg. dry wt.) in 20 mM-sodium formate, 50 mM-tris buffer, pH 7·65, 1 mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

Time (sec.)	Radioactivity (counts/min.)								
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds	Total
7	19	5	39	11	0	0	0	0	74
14	28	16	84	38	0	18	0	52	236
21	8	11	67	8	30	26	0	31	181
38	3	16	45	36	26	40	44	139	349
63	9	12	80	95	73	77	68	392	806
84	11	16	104	142	51	103	103	576	1106
122	11	20	74	144	186	97	281	549	1362

Table 4. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of formate-grown* Pseudomonas AM1 *incubated with* [^{14}C]*bicarbonate*

Sodium [^{14}C]bicarbonate (5·9 μmoles; 140 μc of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (28·3 mg. dry wt.) in 20 mM-sodium formate, 50 mM-tris buffer, pH 7·65, 1 mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

	Radioactivity (counts/min.)									
Time (sec.)	Malate	Glycine	Serine	Glut-amate	Aspar-tate	Citrate	Treha-lose	Phosphoryl-ated compounds	Other com-pounds	Total
4	47	8	0	3	43	0	0	0	20	121
14	82	20	0	7	80	15	0	0	65	269
21	76	29	0	8	145	31	0	0	57	346
27	121	46	0	16	266	62	0	58	50	619
50	54	21	0	40	96	78	0	72	34	395
75	56	15	0	86	186	79	36	114	30	602
107	38	13	0	105	170	78	82	165	47	698

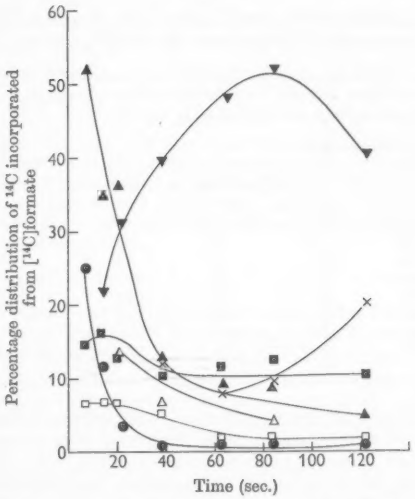

Fig. 4. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]formate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on formate. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

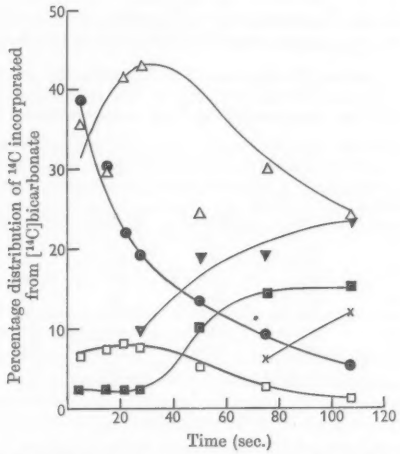

Fig. 5. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]bicarbonate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on formate. ●, Malate; △, aspartate; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

Absence of carboxydismutase in cell-free extracts of the organisms. Cell-free extracts of methanol-grown *Pseudomonas* AM1 and *H. vulgare*, and formate-grown *Pseudomonas* AM1, prepared by crushing the frozen bacteria, were assayed for the presence of carboxydismutase by incubating the extracts with [^{14}C]bicarbonate in the presence or absence of ribulose 1:5-diphosphate (Quayle & Keech, 1960). In no case did the presence of the ribulose diphosphate cause any appreciable stimulation of [^{14}C]bicarbonate fixation. Cell-free extracts of methanol-grown *Pseudomonas* AM1 were also prepared by ultrasonic disintegration and showed no activity in the same assay. The possibility that an inhibitor of carboxydismutase was present in the cell-free extracts was eliminated by the finding that the activity of this enzyme in extracts of formate-grown *Pseudomonas oxalaticus* (Quayle & Keech, 1959*b*) was unaltered in the presence of extracts of either *Pseudomonas* AM1 or *H. vulgare*.

Table 5. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown* Hyphomicrobium vulgare, *incubated with* [^{14}C] *methanol*

[^{14}C]Methanol (37 μmoles; 200 μc of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (120 mg. wet wt.) in 10 mM-methanol, 10 mM-ammonium chloride and 10 mM-sodium phosphate, pH 7·0. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

	Radioactivity (counts/min.)									
Time (sec.)	Malate	Glycine	Serine	Glut-amate	Aspar-tate	Citrate	Phosphoryl-ated compounds	Glycol-late	Other com-pounds	Total
6	3	0	44	0	9	0	0	27	15	98
12	34	9	94	13	58	10	0	42	25	285
18	44	23	101	35	85	5	0	43	23	359
24	74	25	103	48	126	3	0	47	26	452
30	92	25	133	65	178	5	0	72	28	598
47	142	62	155	103	239	7	69	65	56	898
62	149	72	133	211	267	9	205	52	76	1174
100	209	95	154	163	292	8	244	58	267	1490

Table 6. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown* Hyphomicrobium vulgare, *incubated with sodium* [^{14}C]*bicarbonate*

Sodium [^{14}C]bicarbonate (8·1 μmoles; 200 μc of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (120 mg. dry wt.) in 10 mM-methanol, 10 mM-ammonium chloride and 10 mM-sodium phosphate, pH 7·0. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

	Radioactivity (counts/min.)								
Time (sec.)	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Phosphoryl-ated compounds	Other com-pounds	Total
7	138	31	0	0	82	8	0	59	318
15	237	90	23	38	176	34	0	85	683
22	358	151	50	69	242	38	23	140	1071
29	371	216	82	125	380	47	58	200	1479
38	348	283	71	186	413	62	116	225	1704
52	338	333	84	376	510	76	162	305	2184
94	110	369	58	443	227	46	414	339	2006

DISCUSSION

The only known series of reactions which leads to the synthesis of polycarbon compounds from C_1 units is the ribulose diphosphate cycle of carbon dioxide fixation leading to triose phosphate synthesis, discovered by Calvin and his colleagues in photosynthetic tissue (Bassham *et al.* 1954). The cycle has since been found in many species of autotrophic bacteria (see Vishniac, Horecker & Ochoa, 1957; Quayle, 1961). The possibility that microbial growth on organic C_1 compounds might involve an autotrophic type of metabolism, in which the cellular carbon is synthesized from carbon dioxide, had already been foreseen (Bhat & Barker, 1948; van Niel, 1954). That such a mechanism actually operated, and involved the ribulose diphosphate cycle, was demonstrated by Quayle & Keech (1959*a*, *b*) with formate-grown *P. oxalaticus*. Hence, in any investigation of the method of microbial synthesis of cell constituents from C_1 compounds, evidence for the operation of the ribulose diphosphate cycle should first be examined. The experiments reported in this paper eliminate the possibility of its operation in methanol- and formate-grown *Pseudomonas* AM1 and methanol-grown *H. vulgare*. This conclusion is based on the following two criteria: (*a*) the absence of a key enzyme, carboxydismutase, in cell-free extracts of the organisms; (*b*) the course of ^{14}C incorporation into cell constituents follows a totally different pattern from that observed in organisms in which the ribulose diphosphate cycle has been shown to operate. Such organisms show early labelling from $^{14}CO_2$ to occur predominantly in 3-phosphoglyceric acid, non-phosphorylated compounds becoming radioactive at a later time. In both *Pseudomonas* AM1 and *H. vulgare*, radioactivity from $^{14}CO_2$ is initially incorporated into C_4 acids and glycine, whereas phosphorylated compounds appear later. The slope of the curve obtained by plotting against time the percentage of the fixed radioactivity that was con-

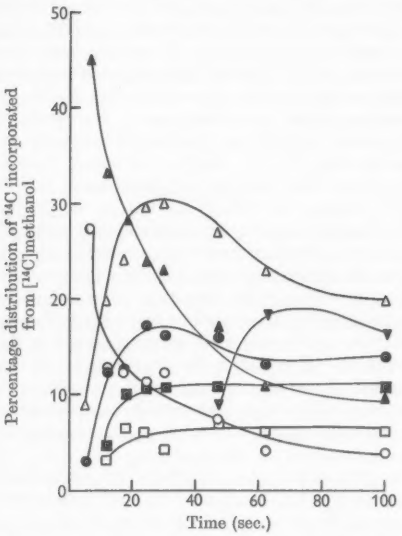

Fig. 6. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]methanol into the constituents of the ethanol-soluble fraction of *Hyphomicrobium vulgare* growing on methanol. ○, Glycollate; ●, malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds.

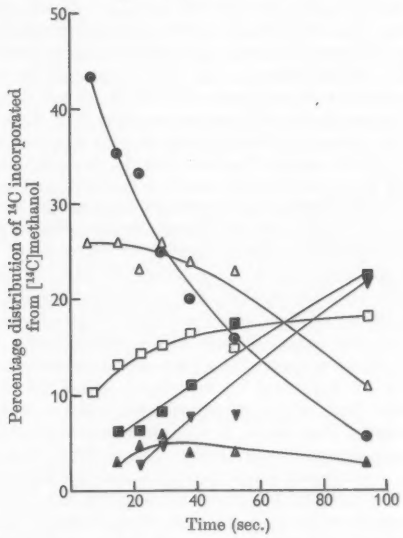

Fig. 7. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]bicarbonate into the constituents of the ethanol-soluble fraction of *Hyphomicrobium vulgare* growing on methanol. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds.

tained in phosphorylated compounds is, in all cases, positive. This shows that these compounds are unlikely to be primary products of $^{14}CO_2$ fixation.

The conclusion that growth of *H. vulgare* on methanol does not involve an autotrophic metabolism is at variance with the conclusion reached by Näveke & Engel (1955). These authors have summarized most of the work published on this organism and, while stating that it is not possible to decide whether the bulk of its carbon is fixed in the form of carbon dioxide or not, they list properties which they consider as constituting indirect evidence for the organism's being regarded as an autotroph: (*a*) slow growth (Mevius, 1953); (*b*) no growth on glucose, sucrose, mannose, or raffinose (Mevius, 1953); (*c*) the presence of $^{14}CO_2$ during growth leads to radioactivity appearing in amino acids isolated from the organism (Mevius, 1953; Näveke, 1957); (*d*) the organism oxidizes formate and oxalate to carbon dioxide and water and releases 85–95 % of the energy as heat (Korte & Engel, 1955). Since the nitrifying bacteria oxidize inorganic nitrogen compounds with the release of 93–95 % of the available energy as heat, the authors state that the similarity in formate or oxalate oxidation by *H. vulgare* leads to the probability that these substrates serve only as energy sources and not as carbon sources.

The first three criteria are very indirect, whereas the difficulties that attend both the measurement of the efficiency of energy utilization in autotrophic and heterotrophic bacteria and the interpretation of the experimental results are such (see Larsen, 1960) as to make an assignment of mode of metabolism based on such values uncertain. In our opinion, the data reported in the present paper argue more directly against the operation of an autotrophic type of metabolism in *H. vulgare* than the above data argue in its favour.

A comparison of the time courses of incorporation of labelled growth substrate or labelled bicarbonate by methanol- or formate-grown *Pseudomonas* AM 1, and methanol-grown *H. vulgare*, shows striking similarities. First, the incorporation of ^{14}C from the labelled substrate as compared with that from [^{14}]bicarbonate is roughly of the same order with each organism, indicating a substantial incorporation of carbon both in organic form and as carbon dioxide. This conclusion is also borne out in *Pseudomonas* AM 1 growing on [^{14}C]methanol by the finding that the specific radioactivity of its cellular material is reduced by 50 % when the culture

is flushed with air–carbon dioxide. If all the carbon fixed by the cell were passing through the stage of carbon dioxide, as an autotrophic metabolism would demand, this reduction might be expected to be of the order of 90–100 %, as was found with formate-grown *P. oxalaticus* (Quayle & Keech, 1959*a*); if most of the cell carbon were not passing through the stage of carbon dioxide, the reduction might be expected to be below about 20 %, as was found with the succinate-grown *Pseudomonas* AM 1 (7 %). A similar type of experiment performed with acetate-grown *Pseudomonas* KB 1 (Kornberg & Quayle, 1958) gave a reduction of 17 % in the specific radioactivity of cellular protein. Secondly, from either [^{14}C]methanol or [^{14}C]formate the compound that contains most of the radioactivity at early times is serine. The percentage contained in serine of the total carbon fixed, when plotted against time, shows a pronounced negative slope, indicating that serine is a primary stable product of methanol or formate incorporation. With *H. vulgare*, glycollate is also indicated as being a primary product in methanol incorporation, although it was not detected in *Pseudomonas* AM 1. Glycine was present to a fairly constant low percentage of the total count fixed throughout the experiments with each tracer. The patterns of incorporation observed with [^{14}]bicarbonate as tracer all show predominant early labelling in C_4 acids such as malate and aspartate and, in contrast with the experiments with [^{14}C]methanol or [^{14}C]formate, glycine contained more radioactivity than did serine. No glycollate was observed in any of the experiments.

The similarities between the results obtained with methanol- and formate-grown *Pseudomonas* AM 1 and methanol-grown *H. vulgare* indicate that an essentially similar pathway of carbon incorporation operates in all three instances. This proposed pathway probably involves substantial incorporation of both reduced carbon and carbon dioxide, although the relative contributions of exchange reactions and net carbon dioxide fixation to the observed carbon dioxide incorporation cannot yet be assessed. For the latter incorporation to be due largely to exchange reactions, these would, however, have to be quite unusually large.

It seems probable that the serine formed from either methanol or formate is made by hydroxymethylation of glycine by the well-known serine hydroxymethylase (Rabinowitz, 1960):

$$\text{Methylenetetrahydrofolate} + \text{glycine} \rightleftharpoons \text{serine} + \text{tetrahydrofolate} \quad (1)$$

If this reaction represents the main step for synthesis of C_3 compounds from C_2 compounds, then a crucial problem lies in the prior synthesis of the C_2 compound, glycine, or its precursors, from C_1 units. Radioactive glycine is present at the earliest times when either [^{14}C]methanol, [^{14}C]formate or [^{14}C]bicarbonate is used and it is tempting to think that it (or possibly glycollate) may be arising from a condensation of two C_1 units derived respectively from organic substrate and carbon dioxide. Such a condensation might be a direct one or the result of a cyclic series of reactions. The rather low level of ^{14}C fixation into glycine, relative to serine, does not support the idea of a direct condensation between two C_1 units. A second alternative, involving a cyclic series of reactions, might result in delayed appearance of radioactivity in glycollate or glycine, but would necessitate rapid labelling in members of such a synthetic cycle. The only other compounds rapidly labelled at the earliest times are malate and aspartate, and hence such C_4 compounds or their derivatives might thus be implicated. A third alternative is that the method of analysis, which deals only with stable, non-volatile compounds that are soluble in aqueous ethanol, is not detecting key intermediates in the metabolism.

At present no other details of the biosynthetic pathway are known, and further information is being sought from studies of the labelling patterns of some of the intermediates which become radioactive after short incubation times, and of the levels of enzymes involved in serine synthesis which are present in the organism.

A similar pathway of synthesis of cell constituents probably operates during the growth of *Pseudomonas* PRL–W 4 on methanol. Kaneda & Roxburgh (1959*a*) showed with this organism that the first labelled compound to be formed from either [^{14}C]methanol or [^{14}C]bicarbonate was serine. No glycine or glycollate was detected. The concentration of tracer used was much less, and far fewer labelled compounds were detected, than in the present series of experiments, which might possibly account for the non-detection of glycine, for instance. Kaneda & Roxburgh (1959*b*) also carried out a series of isotopic experiments, mainly with resting cell suspensions of *Pseudomonas* PRL-W 4, to test the effect of the presence of various combinations of methanol, formaldehyde, formate and carbonate on fixation of either [^{14}C]methanol or $^{14}CO_2$ by the cells. On the basis of these experiments the authors suggested that two simultaneous pathways for the fixation of C_1 compounds exist: carbonate and formate are involved in one pathway, and methanol and formaldehyde in the other. It is presumed that both the formate and formaldehyde are incorporated as serine, possibly by a serine hydroxymethylase system.

It remains to be seen whether microbial growth on methane involves a metabolism broadly similar to that involved in growth on methanol. The isotopic experiments necessary to decide this have not been reported, although some work has been

carried out on *Pseudomonas methanica* by Leadbetter & Foster (1958) which indicates that during growth on methane a considerable proportion of the carbon incorporated by the cell does not pass through a stage readily exchangeable with carbon dioxide. It is thus unlikely that an autotrophic type of metabolism is involved.

SUMMARY

1. A study has been made of the incorporation of carbon from [^{14}C]methanol and [^{14}C]bicarbonate by cultures of *Pseudomonas* AM 1 and *Hyphomicrobium vulgare* grown on methanol, and from [^{14}C]formate and [^{14}C]bicarbonate by cultures of *Pseudomonas* AM 1 grown on formate.

2. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the cells, after incubation for periods up to 2 min., has been analysed by chromatography and radioautography.

3. The first stable products of [^{14}C]methanol or [^{14}C]formate fixation were serine (containing approx. 50 % of the total count fixed) and C_4 dicarboxylic acids such as malate and aspartate. Radioactive glycollate was also observed in *Hyphomicrobium vulgare*. Radioactive phosphorylated compounds were present at the earliest time in methanol-grown *Pseudomonas* AM 1, but only appeared at later times in the other two incubation experiments. Radioactive glycine appeared at early times of incubation, but its proportion of the total count fixed did not exceed 15 % and was less at all times than that of serine. Other compounds which appeared labelled during the incubations included glutamate, citrate and, in *Pseudomonas* AM 1, trehalose.

4. The first stable products of [^{14}]bicarbonate fixation were the C_4 acids, malate and aspartate (containing between them 40–70 % of the total count fixed) and glycine. Other compounds becoming labelled included serine, phosphorylated compounds, glutamate, and, in *Pseudomonas* AM 1, trehalose.

5. The percentage of the total count fixed which was contained in each of the radioactive compounds was plotted against time. The slopes of the curves obtained show that serine is a primary product of [^{14}C]methanol or [^{14}C]formate incorporation (and glycollate in *Hyphomicrobium vulgare*), and that malate or aspartate is a primary product of [^{14}C]bicarbonate incorporation.

6. The specific radioactivity of cellular material obtained from *Pseudomonas* AM 1 grown on [^{14}C]-methanol in air was reduced by one-half on bubbling air–carbon dioxide (99:1, v/v) through the growing culture. This shows that at least 50 % of the carbon incorporated must have passed through the stage of carbon dioxide or a compound in ready equilibrium with it.

7. No carboxydismutase activity has been found in cell-free extracts of the organisms. This fact, together with the other data, shows that an autotrophic metabolism involving the ribulose diphosphate cycle of carbon dioxide fixation cannot be operating.

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Composition of Phospholipids of Rabbit, Pigeon and Trout Muscle and Various Pig Tissues

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Studies on the distribution and structure of the phospholipids in certain mammalian tissues and subcellular components have been reported previously (Gray & Macfarlane, 1958; Gray, 1960*a*, *b*; Macfarlane, Gray & Wheeldon, 1960; Macfarlane, 1961*a*). The object of the present work was to obtain a general picture of the pattern of fatty components in phospholipids in other normal tissues, and to examine further the distribution of choline plasmalogen. This compound is less than 1% of the total phospholipid in brain (Klenk, Debuch & Daun, 1953; Webster, 1960) and a minor component in liver and spleen; but it is a major component (36%) in ram semen and in ox and pig heart (Lovern, Olley, Hartree & Mann, 1957; Gray, 1960*a*; Klenk & Debuch, 1955; Marinetti & Erbland, 1957; Gray & Macfarlane, 1958). It appeared possible that choline plasmalogen is associated directly with a contractile process, though the small amount found in cod muscle (Garcia, Lovern & Olley, 1956) indicated a considerable species difference; Hartree & Mann (1959), on the other hand, suggested that fatty acids derived from plasmalogen participate in the aerobic endogenous metabolism of sperm.

METHODS

The estimation of P, total N, amino N, choline, aldehyde, inositol, fatty acid ester groups, alkali-labile P and phosphomonoester were carried out as in Gray & Macfarlane (1958). The method for the estimation of ethanolamine and serine (Axelrod, Reichental & Brodie, 1953) was modified slightly by substituting an acid hydrolysis and chloroform–methanol-extraction procedure used by Dr C. Long (personal communication) for the usual alkaline hydrolysis. Chromatography of phospholipids on silicic acid-impregnated paper was done in di*iso*butyl ketone–acetic acid–water (40 : 20 : 3, by vol.) at 2° and of glycerides in light petroleum (b.p. 60–80°)–di*iso*butyl ketone (96 : 6, v/v) at room temperature (Marinetti, Erbland & Kochen, 1957). Chromatography on Whatman no. 1 acid-washed paper in butan-1-ol–water–conc. NH_3 (100 : 15 : 2, by vol.; Coulon-Morelec, Faure & Maréchal, 1960) was also used (R_F cardiolipin 0·74; phosphatidic acid 0·48). Chromatography of water-soluble esters obtained by mild hydrolysis was done according to Dawson (1954).

Myofibrils were prepared from rabbit muscle by the method of Perry & Grey (1956); the lipid was compared with that extracted from a portion of the whole muscle from the same animal. Muscle from rainbow trout and pigeons was extracted within 1 hr. after death, and pig tissue obtained from a slaughterhouse within 2 hr.

The extraction of lipid from the tissues was based on the method of Folch, Lees & Sloane-Stanley (1957) and Gray (1960*b*). In general the tissue was blended mechanically with 5 vol. of cold chloroform–methanol (1 : 1, v/v) and re-extracted twice with 1 vol. of chloroform–methanol (2 : 1, v/v). The combined extracts were washed two or three times with 0·2 vol. of salt solution (0·04 M-$MgCl_2$ or 0·05 N-NaCl), and the chloroform solution was dried and evaporated. The residue was dissolved in light petroleum and dialysed according to van Beers, de Iongh & Boldingh (1958) to separate most of the non-P lipid (neutral lipid fraction) from the phospholipid.

The phospholipid was fractionated on silicic acid as in Gray (1960*b*) and neutral lipid by the system of Hirsch & Ahrens (1958). The isolation of the fatty acids from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine and of the fatty acids and aldehydes from the ethanolamine, serine and choline plasmalogen was carried out as described by Gray (1960*b*).

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Table 1. *Yield of total lipid and phospholipid from various tissues*

Weights are calculated for 100 g. of fresh tissue.

Tissue	Total lipid (g.)	Total cholesterol (g.)	Lipid P (mg.)	Phospholipid* (g.)	Phospholipid* (% of total lipid)
Rabbit					
Skeletal muscle 1	2·26	—	20·4	0·51	23
2	—	—	18·0	0·45	—
Pigeon					
Heart muscle	3·86	0·17	102·0	2·55	66
Breast muscle	5·16	0·09	89·0	2·22	43
Trout					
Muscle 1	5·31	0·05	25·4	0·63	12
2	3·72	—	27·8	0·70	19
Pig					
Spleen	2·45	—	49·5	1·24	50
Lung	2·00	0·14	63·6	1·59	79
Kidney	2·90	0·28	93·5	2·34	81
Ram					
Semen	1·37	—	24·8	0·62	45

* Calculated from P content ×25.

The fatty acids as methyl esters and the aldehydes as dimethyl acetals were identified by gas chromatography. The analysis of aldehydes from the plasmalogens of different tissues by gas chromatography described by Gray (1960*c*) made possible the identification of a number of hitherto unknown, naturally occurring aldehydes with branched carbon chains. Further work (Gray, 1961) has shown that these aldehydes belong to either the *iso-* or *anteiso*-series of compounds analogous to the branched-chain acids found in animal tissues, and can be positively identified by their gas-chromatographic behaviour on polar and non-polar stationary phases.

EXPERIMENTAL AND RESULTS

In the fractionation of phospholipid mixtures on silicic acid columns the volume of any one solvent mixture necessary to separate a component varies with the proportion of the component and the nature of the mixture. After passage of chloroform to elute the remaining non-P lipid, the stepwise increase in methanol concentration was not made at stages preset by the volume of solvent to be passed, but at stages determined for individual columns by the separation curve, based on analysis of eluent fractions for P and for amino N, fatty acid esters etc. as appropriate. In general, elution was begun with 2% (v/v) methanol in chloroform, increased in steps of 2% until the cardiolipin fraction was eluted, and then in larger steps, e.g. 10, 15, 20, 25, 35, 50 and 70% (v/v) methanol in chloroform. The usual type of separation curve has been illustrated previously (e.g. Gray, 1960*b*). Eluent fractions were pooled on the basis of the separation curve, supplemented by paper chromatography, and the larger fractions further characterized by analysis for ethanolamine, serine, aldehyde, inositol, alkali-stable P etc. and by paper chromatography. The procedure permitted in general the substantial separation of (1) cardiolipin, (2) phosphatidylinositol, (3) phosphatidylserine or phosphatidylethanolamine, or both, and corresponding plasmalogens, (4) phosphatidylcholine and choline plasmalogen, (5) sphingomyelin. The methods of analysis for various groups and structures present in a phospholipid molecule are, however, not so accurate that the presence of small amounts of other compounds of similar general structure can be excluded. For example, the analyses have not indicated the possible presence in the kephalin and lecithin fractions of the corresponding glycerol ether compound (Carter, Smith & Jones, 1958), but during some recent investigations on the hydrolysis of plasmalogens (Pietruszko & Gray, 1960) evidence was obtained that glycerol ethers may normally occur in small amounts in these fractions. However, it is thought that the computation from the analysis gives a fair picture of the distribution of phosphorus amongst the main components, and the separation curves and detailed analyses for each tissue have been omitted.

The yield of total lipid and lipid P from various tissues is given in Table 1; the composition of the phospholipid in Table 2 and the fatty acid composition in Tables 3–5. Comments on the individual tissues are made below.

Rabbit myofibrils. Choline plasmalogen was present in the myofibrils in slightly higher proportion than in the whole muscle; about 50% of the kephalin was plasmalogen in both whole muscle and myofibrils.

Pigeon-breast muscle. The lipid content was high. About 43% of the total was phospholipid, 2%

cholesterol and the remainder almost entirely triglycerides containing a high proportion of oleic acid. Phosphatidic acid was not detected in the cardiolipin fraction by paper chromatography in butanol–water–conc. ammonia. Choline plasmalogen was present in amounts similar to rabbit skeletal muscle, and was highly unsaturated, about 65 % of the fatty acids being C_{20}–C_{22} polyenoic acids, compared with 7 % of these acids in the phosphatidylcholine fraction (Table 4).

Trout muscle. The total lipid was much higher than that reported for cod and haddock flesh (cf. Garcia *et al.* 1956), mainly owing to an increase in triglyceride content. On fractionation of the neutral lipid on silicic acid, 83 % was recovered in the triglyceride fraction, and 0·9 % as free cholesterol, with only a trace in the cholesterol ester fraction.

The small cardiolipin fraction of the phospholipid was eluted rapidly with 2–4 % methanol in chloroform, and contained some cholesterol and other material. After precipitation with acetone, the product had a ratio P:fatty acid ester:amino N of 1:2·1:0·05, and was still low in P (3 %), but gave a single spot corresponding to cardiolipin on paper. The ethanol-insoluble potassium salt obtained by saponification in ethanolic 0·2N-potassium hydroxide at room temperature, however, contained about 10 % of phosphomonoester by enzymic assay; the content of glycerophosphate P in the salt by Burmaster's (1946) method, after hydrolysis in N-hydrochloric acid at 100° for 10 min., was only 85 % of the total P against 92 % found for authentic cardiolipin ester, although the apparent α-glycerophosphate (42 %) after oxidation with periodate at room temperature was similar to that of cardiolipin (see Gray & Macfarlane, 1958). Some unidentified phospholipid was possibly present. The fatty acid composition of the cardiolipin fraction was significantly different from that of pigeon and ox in the low content of linoleic acid (6 %) and high content (35 %) of saturated acids (Table 3).

Table 2. *Distribution of phospholipids in various tissues*

Values are given as percentage of total lipid P.

	Rabbit muscle		Pigeon muscle			Pig		
Phospholipid	Whole	Myofibrils	Heart	Breast	Trout muscle	Spleen	Lung	Kidney
Cardiolipin	4	6	8	9	4	1	2	4
Phosphatidylinositol	4	4	8	2	2	2	5	3
Phosphatidylethanolamine	24	20	29	31	21	14	12	17
Ethanolamine plasmalogen						13	8	14
Phosphatidylserine				5	4	13	8	7
Serine plasmalogen						3	3	3
Phosphatidylcholine	54	51	43	33	66	33	50	36
Choline plasmalogen	11	14	6	16	< 1·0	2	1	3
Sphingomyelin	—	—	5	3	2	19	12	14
Recovery from column (%)	—	—	95	94	98	98	98	98

Table 3. *Fatty acid composition of triglycerides and cardiolipin*

Values are given as percentage of total fatty acid methyl esters.

		Triglycerides		Cardiolipin			
Fatty acid	Designation	Pigeon breast	Trout muscle	Pigeon breast	Trout muscle	Pig lung	Pig kidney
Myristic	14 : 0	0·3	2·8	Trace	2·0	0·4	—
Palmitic	16 : 0	19·0	20·0	0·4	19·1	5·9	5·8
Stearic	18 : 0	8·4	4·9	2·2	13·5	4·9	1·5
Total normal saturated	—	27·7	27·8	2·6	34·8	11·2	7·3
Palmitoleic	16 : 1	7·0	10·1	0·7	4·1	2·1	3·1
Oleic	18 : 1	52·8	42·3	12·8	44·6	13·8	15·6
Total monoenoic	—	59·8	52·4	13·5	48·7	15·9	18·7
Linoleic*	18 : 2	12·4	3·3	84·0	6·5	67·1	74·0
Eicosatrienoic	20 : 3	—	3·6	—	—	1·5	—
Arachidonic	20 : 4	Trace	1·3	—	—	2·5	—
Eicosapentanoic	20 : 5	—	3·3	—	—		—
Docosapentaenoic	22 : 5	—	2·6	—	1·7	—	—
Docosahexaenoic	22 : 6	—	3·2	—	—	—	—

* Includes any linolenic acid. Small amounts of branched C_{14}, C_{15}, branched C_{17} and C_{19} acids in some fractions have been omitted.

Table 4. *Fatty acid composition of phospholipids of pigeon and trout muscles*

Values are expressed as percentage of total methyl esters.

		Pigeon-breast muscle			Trout-muscle lecithin			
Fatty acid	Designation	Kephalin	Lecithin	Choline plasmalogen	I	II	III	Mean*
Myristic	14 : 0	—	Trace	Trace	—	0·4	2·6	1·6
Palmitic	16 : 0	4·2	24·4	9·2	6·6	29·6	33·2	26·2
Stearic	18 : 0	50·0	17·9	6·5	5·5	3·0	1·7	2·7
Total normal saturated	—	54·2	42·3	15·7	12·1	33·0	37·5	31·5
Palmitoleic	16 : 1	0·2	1·8	0·5	0·9	—	4·6	2·9
Oleic	18 : 1	4·2	22·6	8·7	8·2	8·7	12·8	11·0
Total monoenoic	—	4·4	24·4	9·2	9·1	8·7	17·4	13·9
Linoleic	18 : 2	7·6	24·1	9·7	—	0·3	4·6	1·6
Eicosatrienoic	20 : 3	5·5	1·6	7·9	2·0	—	—	0·4
Arachidonic	20 : 4	26·9	3·8	25·6	12·0	2·4	2·9	10·9
Eicosapentaenoic	20 : 5	1·2				6·7	8·2	
Total C_{20} polyenoic	—	33·6	5·4	33·5	14·0	9·1	11·1	11·3
Docosapentaenoic	22 : 5	—	2·0	24·4	3·9	2·2	3·0	3·0
Docosahexaenoic	22 : 6	—	0·5	7·5	61·0	43·4	29·3	38·5
Total C_{22} polyenoic	—	Nil	2·5	31·9	64·9	45·6	32·3	41·5

* Weighted mean. Includes any linolenic acid. Small amounts of branched C_{16}, branched C_{17} and C_{17} acids in some fractions have been omitted.

The kephalin fraction contained about 11 % of plasmalogen and was highly unsaturated (double bonds/molecule of P, 5·6); possibly in consequence of this, considerable decomposition occurred in this fraction on storage before saponification, and analyses for fatty acids were therefore not done.

The lecithin fraction contained less than 1 % of choline plasmalogen and was very highly unsaturated. Three successive fractions I, II and III from the silicic acid column, containing 19, 20 and 60 % of the lecithin P, had a P content 3·8–4·0 % and a ratio N:P of 0·96–1·08:1; these fractions gave a single spot on paper chromatography, and 96 % of the P was labile to mild alkali and acid. On saponification by refluxing for 2 hr. in 0·5N-potassium hydroxide in 95 % ethanol, 93 % of the P became water-soluble, all of which was present as glycerophosphate P by Burmaster's (1946) method. The three lecithin fractions were analysed separately for fatty acids (Table 4); nearly 40 % of the acids were C_{22} hexaenoic acids, and less than 2 % linoleic acid.

Pig spleen, lung and kidney. Approximately 80 % of the total lipid in lung and kidney and 50 % in spleen was phospholipid. The three tissues were generally similar in composition with no particular phospholipid conspicuous by its absence (Table 2).

Compared with that in heart tissue the amount of cardiolipin found was low, especially in spleen. A fatty acid analysis was not carried out on the cardiolipin from this source because the small fraction obtained was contaminated with too much phosphatidylethanolamine and phosphatidylserine. The ratio of phosphatidylethanolamine to phosphatidylserine varied from approximately 1:1 in spleen to 3:2 in lung and 5:2 in kidney. The spleen both from pig and ox (Gray, 1960*b*) appears to be one of the richest natural sources of phosphatidylserine. Ethanolamine plasmalogen was the major aldehydogenic compound in all three tissues with serine plasmalogen (3 %) and choline plasmalogen (3–1 %) present as minor components. Lecithin was as usual the major tissue phospholipid and sphingomyelin also occurred in substantial amounts (12–19 %). Phosphatidylinositol was present in minor quantities, though in lung its proportion (5 %) was greater than that of cardiolipin, serine plasmalogen or choline plasmalogen.

The fatty acid distribution in the lecithin, kephalin, choline plasmalogen and kephalin plasmalogen of the three tissues differed in a number of ways (Table 5). Spleen had the highest degree of unsaturation (expressed as double bonds per molecule of fatty acid) and lung the lowest. A large proportion of the acids from the kephalin plasmalogen fractions were C_{20} and C_{22} polyenoic compounds, that in spleen kephalin plasmalogen being exceptionally high (75·6 % of total acids). The kephalin plasmalogen and choline plasmalogen fatty acids were more unsaturated than those of the corresponding kephalin and lecithin, with the exception of lung choline plasmalogen, which contained 71 % of saturated acids and only 24 % of a monounsaturated acid.

The aldehydes (Table 6) from the choline plasmalogen and kephalin plasmalogen of lung and the

Table 5. *Fatty acid composition of different phospholipids from pig spleen, lung and kidney*

Values are expressed as percentage of total methyl esters

Acid	Designation	Kephalin			Kephalin plasmalogen			Lecithin			Choline plasmalogen		
		Spleen	Lung	Kidney	Spleen	Lung	Kidney	Spleen	Lung	Kidney	Spleen	Lung	Kidney
Myristic	14 : 0	0·1	0·2	Trace	Trace	—	0·1	0·4	1·3	0·2	0·4	2·0	0·1
Palmitic	16 : 0	3·8	9·1	11·0	3·3	4·3	4·9	30·0	47·1	29·0	27·2	64·3	18·8
Stearic	18 : 0	38·8	26·8	45·0	13·4	9·1	17·3	14·7	8·5	16·0	7·9	5·1	8·1
Total normal saturated		42·7	36·3	56·0	26·7	13·4	22·3	45·1	56·9	45·2	35·5	71·4	27·0
Total odd-numbered saturated		0·4	1·1	0·6	0·8	0·7	0·2	1·0	1·0	0·5	0·6	1·2	0·1
Total branched-chain saturated		0·4	0·6	0·2	Trace	0·6	Trace	0·8	0·7	0·5	0·4	0·7	0·1
Palmitoleic	16 : 1	0·4	0·9	0·3	0·3	0·9	0·2	0·8	2·8	0·7	2·0	3·6	0·3
Oleic	18 : 1	18·2	41·1	28·5	8·9	21·5	9·9	24·8	26·2	26·3	12·9	20·4	26·2
Total monoenoic		18·6	42·0	28·8	9·2	22·4	10·1	25·6	29·0	27·0	14·9	24·0	26·5
Linoleic	18 : 2	3·1	4·8	7·0	3·1	4·1	6·3	9·5	6·3	18·0	7·6	2·6	18·9
Linolenic	18 : 3	Trace	Trace	—	—	—	0·3	Trace	0·5	Trace	—	Trace	0·8
Total C_{18} di- and tri-enoic		3·1	4·8	7·0	3·1	4·1	6·6	9·5	6·8	18·0	7·6	2·6	19·7
Eicosaenoic	20 : 1	—	0·4	—	—	Trace	—	—	—	—	—	—	—
Eicosadienoic	20 : 3	7·3	2·4	5·7	11·4	—	5·7	3·9	0·9	1·6	10·2	—	4·4
Arachidonic	20 : 4	16·8	9·3		43·9	39·4	46·5	12·3	4·9	6·9	23·8	0·3	16·3
Eicosapentaenoic	20 : 5	2·2	Trace	1·1	5·0	2·5	3·0		—	Trace	3·1	—	2·9
Total C_{20} polyenoic		26·3	12·1	6·8	60·3	41·9	54·2	16·2	5·8	8·5	37·1	0·3	23·6
Docosatetraenoic	22 : 4	3·2	—	—	6·0	4·7	—	—	—	—	1·7	—	—
Docosapentaenoic	22 : 5	2·5	2·4	Trace	6·4	5·4	2·6	0·7	—	0·6	1·8	—	1·8
Docosahexaenoic	22 : 6	1·5	0·5	—	2·9	1·1	2·9	—	—	—	0·4	—	0·7
Total C_{22} polyenoic		7·2	2·9	Trace	15·3	11·2	5·5	0·7	—	0·6	3·9	—	2·5
Average no. of double bonds per fatty acid molecule		1·6	1·1	0·7	3·2	2·5	2·7	1·1	0·65	0·98	1·9	0·3	1·7

Table 6. *Aldehyde composition of plasmalogens from pig, pigeon and trout*

Values are expressed as percentage of total dimethyl acetals

Aldehyde	Designation	Pig spleen		Pig lung		Pig kidney		Pigeon muscle		Trout muscle
		Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Kephalin plasmalogen
Lauraldehyde	12 : 0	—	—	—	—	—	—	—	1·7	—
Myristaldehyde	14 : 0	Trace	Trace	0·5	0·4	3·0	Trace	—	0·3	—
12-Methyltridecanal / 11-Methyltridecanal	br. 14 : 0	Trace	0·1	Trace	Trace	—	Trace	0·1	—	—
Pentadecanal	15 : 0	0·9	0·3	1·8	1·3	3·9	3·0	1·0	2·7	11·0
13-Methyltetradecanal	*iso*-br. 15 : 0	Trace	—	Trace	0·8	3·8	3·4	—	—	0·5
12-Methyltetradecanal	*anteiso*-br. 15 : 0	Trace	0·1	0·8	0·3	Trace	1·4	0·5	0·2	—
Palmitaldehyde	16 : 0	66·6	41·3	81·5	80·0	81·5	50·0	81·5	43·0	66·5
Palmitoleylaldehyde	16 : 1	1·0	0·6	—	—	—	—	—	2·4	—
14-Methylpentadecanal / 13-Methylpentadecanal	br. 16 : 0	0·3	0·7	0·2	0·2	0·4	8·2	—	0·9	—
Heptadecanal	17 : 0	2·4	3·0	1·9	2·1	—	5·8	0·7	2·6	Trace
	17 : 1	4·4	1·7	—	—	—	—	—	—	—
16-Methylheptadecanal	*iso*-br. 17 : 0	0·8	0·4	0·4	0·7	0·5	Trace	—	—	4·3
15-Methylheptadecanal	*anteiso*-br. 17 : 0	1·7	0·9	0·8	1·1	1·6	1·2	—	Trace	—
Stearaldehyde	18 : 0	12·2	39·6	5·4	6·4	3·1	11·6	10·5	36·7	3·7
Oleylaldehyde	18 : 1	6·7	12·2	5·1	6·3	1·6	4·5	4·6	9·9	10·7
iso-Oleylaldehyde	*iso* 18 : 1	2·7	5·5	1·2	Trace	Trace	0·8	1·3	—	—
Linoleylaldehyde	18 : 2	0·4	1·0	0·4	0·6	0·4	—	—	—	—
17-Methyloctadecanal	*iso*-br. 19 : 0	—	—	—	—	—	—	—	—	—
16-Methyloctadecanal	*anteiso*-br. 19 : 0	—	—	—	—	—	10·3	—	—	3·4
Eicosanal	20 : 0	—	—	—	—	—	—	—	0·13	Trace

choline plasmalogen of kidney had a high proportion (80%) of palmitaldehyde. The spleen aldehydes contained a higher proportion of unsaturated aldehydes, mainly oleylaldehyde and linoleylaldehyde but including some monounsaturated heptadecanal, than lung or kidney. The kidney aldehydes on the other hand had a higher proportion of branched-chain compounds. The aldehydes from the kidney kephalin plasmalogen were peculiar in the amounts of branched C_{19} and branched C_{16} (8%) compounds present.

DISCUSSION

It might be expected that differences in family, species and diets would be reflected in variation in the nature and proportion of the different phospholipids and in their fatty acid composition. The analyses reported here for various tissues indicate little difference between beast, bird and fish in the nature of the phospholipids in the same kind of tissue, except for the virtual absence of choline plasmalogen from trout muscle. Moreover there is comparatively little difference in the fatty acid composition of the same phospholipid from the same tissue of different species in the proportions of saturated and polyenoic acids: the most notable exception to this is that cardiolipin from trout muscle contained little linoleic acid and a high proportion of saturated acids, and the lecithin contained a high proportion of docosahexaenoic acid.

The most substantial differences in fatty acid composition in these tissues appear to lie between the different kinds of phospholipid irrespective of tissue or species. The results of the present and previous work are presented graphically in Fig. 1 to show the C_{16} and C_{18} saturated acids, the mono- and di-enoic acids, and the C_{20} and C_{22} polyenoic acids in the triglycerides, lecithin and kephalin fractions. The preponderance of stearic acid as the saturated acid in kephalin and kephalin plasmalogen and of palmitic acid in lecithin and choline plasmalogen, noted previously (Gray, 1960*b*), holds except for liver lecithin, which both in the ox and the rat contains more stearic than palmitic acid. The distribution patterns of other fatty acids were far less predictable, though certain trends were indicated; the proportions of C_{20} polyenoic acids, especially arachidonic acid, and in general the C_{22} polyenoic acids in the kephalins were higher than in the corresponding lecithins and the lecithins contained a higher proportion of linoleic acid. Similar differences in the distribution of the unsaturated acids occur between the kephalin plasmalogens and the choline plasmalogens.

The tissue triglycerides analysed, though few in number, are remarkably similar to each other and to those reported for normal aorta (Böttcher, Boelsma-van Houte, Romeny-Wachter, Woodford & van Gent, 1960) and differ substantially from the kephalins in that palmitic acid is the preponderant saturated acid, and from both kephalins and lecithins in the low content of linoleic and polyenoic acid. It is clear that there is a considerable selectivity towards the fatty components in these compounds, as well as in cardiolipin and phosphatidylinositol, which should be borne in mind in considering the general validity of routes for the interconversion of tri- and di-glycerides and phospholipids—for example, in the biosynthesis of lecithin by transmethylation of phosphatidylethanolamine.

Saturated fatty acids with an odd number of carbon atoms and branched-chain fatty acids with both even and odd carbon numbers were minor components of all the tissues. The most commonly occurring, and very often the only members of these series, were the *anteiso* C_{15} and C_{17} acids, 12-methyltetradecanoic and 14-methylhexadecanoic acid. It seems possible that these branched fatty acids are of bacterial origin, for it is now known that they are the predominant or major kind of

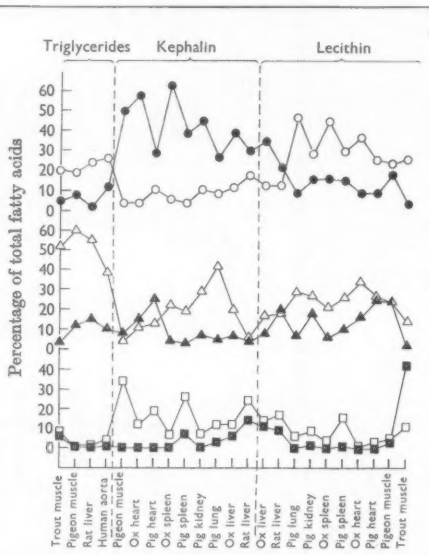

Fig. 1. Relative proportions of certain fatty acids occurring in the triglyceride, kephalin and lecithin fractions from different tissues. ○, Palmitic acid; ●, stearic acid; △, monoenoic C_{16} and C_{18} acids; ▲, dienoic C_{18} (including traces of trienoic C_{18} acid); □, polyenoic C_{20} acids; ■, polyenoic C_{22} acids. Values for triglycerides of human aorta are from Böttcher, Boelsma-van Houte, Romeny-Wachter, Woodford & van Gent (1960).

fatty acid in certain bacteria such as *Sarcina, Bacillus subtilis* and *Micrococcus lysodeikticus* (Akashi & Saito, 1960; Macfarlane, 1961*b*). James, Webb & Kellock (1961) note the occurrence of heptadecanoic acid as well as 10-hydroxystearic acid in the faecal lipids of subjects with steatorrhoea and discuss the possibility of synthesis by micro-organisms.

The distribution of palmitaldehyde (C_{16}) and stearaldehyde (C_{18}) in the plasmalogens of different tissues was similar to that of palmitic acid and stearic acid in the corresponding diacyl compounds (Gray, 1960*c*). The main difference was that, although there was more stearaldehyde in kephalin plasmalogen than in choline plasmalogen, palmitaldehyde was a large and usually the major normal saturated component in both. Saturated aldehydes with an odd number of carbon atoms and branched-chain aldehydes were minor components of all the tissues examined in this recent work. The C_{15} and C_{17} normal and branched-chain (both *iso* and *anteiso*) compounds occurred most frequently though small amounts of branched C_{14} and C_{16} aldehydes were often present. The aldehydes from pig-kidney and trout-muscle kephalin plasmalogen also included a branched-chain C_{19} aldehyde which was probably 16-methyloctadecanal.

In general aldehydes isolated from the plasmalogens of ox tissues (Gray, 1960*c*) had a far greater proportion of branched-chain compounds than those isolated from pig tissues (Table 7). In ox-spleen choline plasmalogen half of the total aldehydes were branched compounds, mainly 13-methylpentadecanal, but in pig spleen only 3% of the total aldehydes were branched compounds. The proportion of aldehydes with odd-numbered carbon chains was small (2–11%) varying only slightly from tissue to tissue and species to species. The proportions of unsaturated aldehydes varied more in pig tissues than in ox tissue.

The data so far obtained on the fatty acid and aldehyde composition of tissue phospholipids of different species suggests further limitations (Gray, 1960*b*) to the enzyme systems which are involved in the biosynthesis and metabolism of the plasmalogens. In all the sources so far examined the range of aldehydes (C_{14}–C_{18}) present is narrower than that of the fatty acids (C_{14}–C_{22}). Presumably the enzyme responsible for producing the typical $\alpha\beta$-unsaturated ether linkage in the plasmalogen molecule will not in general act on compounds with a carbon chain longer than 18 carbon atoms in the α-position of the glycerol moiety. Of course it is also possible that the high degree of unsaturation normally present in the C_{20} and C_{22} carbon chains and not the chain length prevents the enzyme from functioning.

SUMMARY

1. The lipid was extracted from pig spleen, lung and kidney, pigeon, trout and rabbit muscle, and the phospholipid fractionated quantitatively on silicic acid columns.

2. The distribution of the lipid phosphorus amongst cardiolipin, phosphatidylinositol, ethanolamine-, serine- and choline-containing diacyl

Table 7. *Amounts of branched-chain aldehydes, normal saturated odd-numbered carbon chain aldehydes and unsaturated aldehydes present in plasmalogens from different tissues*

Values for each class of aldehyde are expressed as a percentage of the total aldehydes.

	Branched-chain	Normal saturated odd-numbered carbon chain	Unsaturated
Choline plasmalogen			
Ox spleen	50·4	8·3	1·2
Ox heart	15·9	4·9	1·7
Ox liver	56·5	6·1	10·2
Pig heart	2·9	3·3	4·8
Pig spleen	2·9	3·3	15·2
Pig lung	2·2	2·0	6·8
Pig kidney	6·3	3·9	2·1
Pigeon muscle	0·6	1·7	5·9
Ram semen	17·7	8·9	2·4
Kephalin plasmalogen			
Ox spleen	9·1	6·5	8·7
Ox heart	7·9	7·1	6·9
Ox liver	52·4	8·6	5·0
Pig heart	4·4	4·7	10·5
Pig spleen	2·2	3·3	20·9
Pig lung	3·1	3·4	6·9
Pig kidney	23·3	8·8	5·2
Pigeon muscle	1·1	5·3	12·3
Trout muscle	8·2	11·0	10·7

and plasmalogen compounds and sphingomyelin was computed from the analysis of the fractions.

3. Choline plasmalogen was present in similar proportions in the whole muscle and myofibrils of rabbit muscle, but was virtually absent from trout muscle.

4. The constituent fatty acids and aldehydes from the separated phospholipids and some triglyceride fractions were identified and estimated by gas chromatography.

5. In general the results showed a preponderance of C_{18} saturated acids or aldehydes, and of polyenoic acids, in the kephalins, and of the C_{16} saturated compounds in phosphatidylcholine and choline plasmalogen, with the exception of liver and trout-muscle lecithins, which contained large amounts of C_{22} acids.

6. The fatty aldehydes fall in a narrower range (C_{14}–C_{18}) than the fatty acids (C_{14}–C_{22}), and included both unsaturated and branched-chain aldehydes, which varied considerably in proportion in different species.

7. The different fatty acid composition of lecithin, kephalin and triglyceride components of various tissues should be considered in possible routes of biosynthesis.

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Alternative Pathways of Carbohydrate Metabolism in Foetal and Adult Tissues

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The recognition of pathways of carbohydrate metabolism other than the classical Embden–Meyerhof glycolytic sequence has stimulated efforts to clarify the reaction mechanisms involved and to evaluate the relative importance of these alternative pathways in different tissues. Evidence about the fractions of glucose metabolized via the glycolytic sequence and via the hexose monophosphate pathway has been obtained from experiments in which the differential utilization of specifically labelled glucose or other carbohydrate intermediates has been measured. There is as yet

no complete agreement as to the most valid method of calculating, from such experiments, the relative amount of carbohydrate metabolized by the two pathways. A widely used method compares the rates at which C-1 and C-6 of glucose are converted into carbon dioxide. C-6 of glucose is converted into carbon dioxide by glycolysis and the tricarboxylic acid cycle. C-1 of glucose is converted into carbon dioxide both by this pathway and by the hexose monophosphate pathway. C-1 of glucose is removed as carbon dioxide in the oxidative decarboxylation of 6-phosphogluconate to give ribulose 5-phosphate and carbon dioxide, the second enzymic reaction of the pathway. The ratio of the rates at which C-6 and C-1 are metabolized to carbon dioxide gives an estimate of the importance of the two pathways. If all the glucose is metabolized via glycolysis and the tricarboxylic acid cycle the ratio will be unity. If a large fraction of glucose is metabolized via the hexose monophosphate pathway the ratio will be less than unity and will approach zero. Some of the difficulties involved in interpreting such results have been discussed in detail by de Duve & Hers (1957) and Katz & Wood (1960).

Both the dehydrogenation of glucose 6-phosphate to 6-phosphogluconate and the dehydrogenation of 6-phosphogluconate to ribulose 5-phosphate require triphosphopyridine nucleotide as hydrogen acceptor, whereas the dehydrogenations in glycolysis are diphosphopyridine nucleotide-linked. The finding that reduced triphosphopyridine nucleotide is specifically required for certain steps in the synthesis of fatty acids, steroids, purines, amino acids and certain other metabolically important compounds has led to the suggestion that the major role of the hexose monophosphate pathway is to provide reduced triphosphopyridine nucleotide for these synthetic reactions (Siperstein, 1958). We decided therefore to compare the relative rates of glycolysis and hexose monophosphate pathway in foetal and adult tissues, and particularly in those tissues such as the adrenal and ovary in which the synthesis of steroids may be occurring at a high rate. Evidence of high activity of the hexose monophosphate pathway in human foetal adrenal has been presented by Villee, Engel & Villee (1959).

MATERIALS AND METHODS

Samples of liver, brain, heart, lung, kidney, adrenal and skeletal muscle were obtained from foetuses within a few minutes of delivery. Human foetuses were removed from the uterus either by miniature caesarean section or by vaginal hysterotomy. Foetal tissues were obtained from rat and rabbit foetuses by dissecting open the uterus and removing the foetuses. Tissues were obtained from adult dogs and human beings under anaesthesia in the course of operations performed for a variety of purposes. All tissues were placed in ice-cold 0·9% sodium chloride for transfer to the laboratory. Tissue slices, 0·3 mm. thick, were cut with a Stadie–Riggs microtome and a portion was taken for analysis of glycogen content. Tissue slices (about 200 mg.) were weighed on a Roller–Smith torsion balance and placed in Warburg vessels in a medium containing/ml.: 40 μg.atoms of K^+, 80 μg.atoms of Na^+, 10 μg.atoms of Mg^{2+}, 100 μg.atoms of Cl^- and 40 μmoles of phosphate, with an initial pH of 6·9. [1-^{14}C]Glucose or [6-^{14}C]glucose was placed in the side arm of the Warburg vessel and was tipped in at zero time, after the vessels had been gassed with oxygen or nitrogen for 7 min. The final concentration of the glucose in the incubation medium was 11·1 mM. The slices were incubated for 1 hr. unless otherwise stated at 37° either in 3·0 ml. of medium in a Warburg flask of about 15 ml. capacity or in 1·5 ml. of incubation medium in a Warburg flask of about 7 ml. capacity.

After the incubation period the tissue slices were removed, blotted dry on filter paper, weighed, and homogenized in chloroform–methanol (1:1, v/v) for extraction of lipids by the method of Folch, Lees & Sloane-Stanley (1957). The total lipid extract was evaporated to dryness and weighed, and a portion was transferred to a stainless-steel planchet for ^{14}C analysis. Another portion of the tissue slice after incubation was analysed for glycogen content by the method of Walaas & Walaas (1950).

Portions of the incubation medium were analysed for glucose (Nelson, 1944) and lactate (Barker & Summerson, 1941). To other portions of the incubation medium carrier glucose was added and glucose phenylosazones were prepared and purified for ^{14}C analyses (Villee, 1953). The respiratory carbon dioxide was trapped in alkali placed in the centre of the Warburg flask (0·2 ml. of 5% sodium hydroxide, carbon dioxide-free). After the incubation this solution was washed into centrifuge tubes containing water, and the carbon dioxide was precipitated as barium carbonate by the addition of barium chloride at pH 10·5. The barium carbonate was washed with water and ethanol and quantitatively transferred to stainless-steel planchets for ^{14}C analysis. ^{14}C analyses were made with a window-less proportional-flow counter and were corrected for self-absorption. Each sample was counted long enough to reduce the probable error of counting to less than 3%.

RESULTS

The results of experiments with slices from human foetal tissues are summarized in Table 1. The tissues were from foetuses ranging in weight from 35 to 1385 g. and in crown–rump length from 6·5 to 25·5 cm. There was no significant trend in the relative rates of metabolism of glucose by these two pathways with age in the tissues tested, and all the observations for a given tissue were averaged. From the ratios of conversion of glucose carbon to carbon dioxide (column 4) it would appear that the activity of the hexose monophosphate pathway is very small or absent in cerebral cortex but present in medullary tissue from the brain. Other tissues have various degrees of phosphogluconate activity as judged by their

Table 1. *Metabolism of* [1-^{14}C]- *and* [6-^{14}C]-*glucose to carbon dioxide and to lipid by human foetal tissues incubated under aerobic conditions*

The values are mean ± S.E. expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Tissue	No. of expts.	Glucose carbon→CO_2			Glucose carbon→lipid		
		C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
Liver	17	0·61 ±0·066	0·15 ±0·022	0·26 ±0·042	0·14 ±0·033	0·21 ±0·043	1·50 ±0·25
Lung	5	0·067 ±0·0071	0·029 ±0·0033	0·43 ±0·047	0·037 ±0·0046	0·14 ±0·031	3·9 ±0·52
Heart	8	0·18 ±0·024	0·11 ±0·017	0·59 ±0·067	0·020 ±0·0030	0·020 ±0·0021	1·00 ±0·13
Brain, cerebral cortex	8	0·48 ±0·051	0·44 ±0·049	0·92 ±0·088	0·088 ±0·0092	0·069 ±0·0074	0·79 ±0·081
Brain, medulla	7	0·22 ±0·031	0·096 ±0·011	0·44 ±0·08	0·11 ±0·014	0·099 ±0·013	0·91 ±0·088
Kidney	8	0·26 ±0·030	0·044 ±0·008	0·17 ±0·014	0·068 ±0·010	0·10 ±0·014	1·48 ±0·061
Diaphragm	4	0·11 ±0·016	0·036 ±0·006	0·33 ±0·048	0·017 ±0·0040	0·018 ±0·0031	1·07 ±0·14
Adrenal	9	1·00 ±0·12	0·055 ±0·010	0·055 ±0·006	0·11 ±0·011	0·14 ±0·015	1·36 ±0·18
Adrenal tumour	2	0·55 ±0·061	0·048 ±0·008	0·087 ±0·02	0·14 ±0·031	0·16 ±0·026	1·12 ±0·12

characteristic ratios of glucose C-6 to glucose C-1 metabolized to carbon dioxide. The lowest ratio of C-6/C-1 conversion into carbon dioxide was found in the foetal adrenal gland. A similarly low ratio was found in an adrenal tumour from a 6-year-old boy. Other metabolic properties of the foetal adrenal and of this tumour will be presented elsewhere (Villee, Engel, Loring & Villee, 1961). In certain of these foetal tissues the absolute rate of conversion of glucose C-6 into lipid exceeds the rate of its conversion into carbon dioxide. Foetal liver, lung, kidney and adrenal have a very high rate of lipogenesis (Villee, Hagerman, Holmberg, Lind & Villee, 1958). At the earliest age tested, about 10–12 weeks gestational age, each of these tissues has established a characteristic pattern of carbohydrate metabolism which yields reproducible ratios for the conversion of C-6/C-1 into carbon dioxide and of C-6/C-1 into lipid. The ratios do not change as the foetus develops to term.

The effects of anoxia on the relative rates of metabolism of C-1 and C-6 of glucose to carbon dioxide and to lipid were measured by incubating tissue slices in vessels that were flushed with nitrogen instead of oxygen. The fact that there was no pressure change during the period of incubation was taken as evidence of complete anaerobiosis, i.e. that there was no oxygen in the vessel to be utilized. With each tissue, the ratio of glucose C-6 to C-1 metabolized to carbon dioxide was lower in nitrogen than in oxygen (Table 2), indicating that under anaerobic conditions the fraction of glucose metabolized via the hexose monophosphate pathway is larger than the fraction metabolized by that pathway aerobically. Under anaerobic conditions the hexose monophosphate pathway would be limited by the concentration of oxidized TPN (required as cofactor for glucose 6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase) and by the mechanisms available for reoxidizing reduced TPN. Even under aerobic conditions tissues contain relatively little oxidized TPN; most of the TPN is present in the reduced state (Glock & McLean, 1955), thus the second factor is probably more important than the first. Although the hexose monophosphate pathway has been termed the 'direct oxidative pathway' of glucose metabolism, it is depressed by anaerobiosis less than are the enzymes of the Krebs cycle, which are involved in converting C-6 of glucose into carbon dioxide. The synthesis of lipid from both C-1 and C-6 of glucose is depressed by anaerobiosis but to a lesser extent than is the conversion of glucose carbon into carbon dioxide. When liver slices were incubated with air as the gas phase instead of oxygen, the metabolism of both C-1 and C-6 to CO_2 and to lipid was lowered.

The effects of ascorbic acid on glucose metabolism by foetal tissues are summarized in Table 3. The addition of ascorbic acid (30 μmoles/vessel) had no effect on the metabolism of glucose C-1 or C-6 to lipid or on the rate of metabolism of glucose C-6 to carbon dioxide. It did, however, have a marked effect on the rate of conversion of glucose C-1 into

carbon dioxide. The increases ranged from 40 % in liver to fivefold in lung, and was evident in all tissues tested. It seemed possible that the ascorbic acid might be acting by oxidizing $TPNH_2$ and that the increases observed were actually the results of an increased concentration of TPN. The direct addition of 2 μmoles of TPN/vessel produced a slight increase in the conversion of glucose C-1 into CO_2, but the increase was not as great as that observed with ascorbic acid (Table 4). In contrast, the addition of 2 μmoles of DPN decreased the conversion of both glucose C-1 and C-6 into carbon dioxide and into lipid. Since the pyridine nucleotides added *in vitro* might have difficulty in entering the cell this small effect with added TPN may not rule out the possibility that ascorbic acid reoxidizes $TPNH_2$ and makes oxidized TPN available for metabolism of glucose 6-phosphate via

Table 2. *Metabolism of* [1-^{14}C]- *and* [6-^{14}C]-*glucose to carbon dioxide and to lipid by human foetal tissues incubated under anaerobic conditions*

The values are mean ± S.E. expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Tissue	No. of expts.	Glucose carbon→CO_2			Glucose carbon→lipid		
		C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
Liver	9	0·051 ±0·009	0·0096 ±0·0023	0·19 ±0·031	0·022 ±0·0060	0·024 ±0·0065	1·07 ±0·16
Heart	3	0·062 ±0·033	0·0069 ±0·0031	0·11 ±0·044	0·021 ±0·0024	0·022 ±0·0045	1·0 ±0·17
Brain, cerebral cortex	3	0·048 ±0·0088	0·035 ±0·0074	0·73 ±0·081	0·024 ±0·0018	0·012 ±0·0011	0·5 ±0·03

Table 3. *Effects of the addition of* 10 mM-*ascorbic acid on the metabolism of glucose to carbon dioxide and lipid by human foetal tissues*

The values are mean ± S.E. of four experiments and are expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Tissue	Ascorbic acid	Glucose carbon→CO_2			Glucose carbon→lipid		
		C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
Liver	−	0·87 ±0·091	0·18 ±0·022	0·20 ±0·045	0·072 ±0·015	0·17 ±0·033	2·34 ±0·46
	+	1·40 ±0·11	0·24 ±0·029	0·17 ±0·037	0·082 ±0·018	0·16 ±0·030	1·99 ±0·26
Lung	−	0·067 ±0·0071	0·029 ±0·0033	0·43 ±0·047	0·035 ±0·0044	0·085 ±0·0092	2·43 ±0·48
	+	0·36 ±0·039	0·016 ±0·0023	0·045 ±0·020	0·039 ±0·0047	0·080 ±0·010	2·05 ±0·41
Brain, medulla	−	0·33 ±0·045	0·11 ±0·014	0·33 ±0·052	0·092 ±0·013	0·098 ±0·013	1·06 ±0·09
	+	1·01 ±0·11	0·078 ±0·0097	0·07 ±0·028	0·082 ±0·012	0·097 ±0·012	1·19 ±0·11
Kidney	−	0·22 ±0·028	0·040 ±0·0082	0·18 ±0·016	0·063 ±0·009	0·090 ±0·011	1·43 ±0·13
	+	0·56 ±0·074	0·030 ±0·0066	0·054 ±0·022	0·074 ±0·009	0·084 ±0·011	1·13 ±0·11

Table 4. *Effects of the addition of cofactors on the metabolism of glucose to carbon dioxide and lipid by human foetal liver*

The values are mean ± S.E. of four experiments and are expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Cofactor added	Glucose carbon→CO_2			Glucose carbon→lipid		
	C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
None	0·56 ±0·072	0·10 ±0·016	0·19 ±0·039	0·11 ±0·024	0·13 ±0·025	1·12 ±0·13
TPN, 2 μmoles	0·64 ±0·075	0·11 ±0·016	0·17 ±0·038	0·11 ±0·023	0·13 ±0·025	1·14 ±0·13
DPN, 2 μmoles	0·48 ±0·055	0·059 ±0·008	0·12 ±0·022	0·077 ±0·014	0·093 ±0·017	1·20 ±0·15

glucose 6-phosphate dehydrogenase. When liver homogenates are used instead of liver slices, the addition of TPN does increase the rate of the hexose monophosphate pathway and the addition of DPN increases the rate of glycolysis (Siperstein, 1958).

The effects of the addition of unlabelled potassium pyruvate (10 μmoles/ml.) and of D-2-deoxyglucose (6·1 μmoles/ml.) to the system are summarized in Table 5. The pyruvate added to the incubation medium would dilute the intracellular pool of labelled pyruvate derived largely from hexose and then decrease the rate of conversion of labelled glucose into acetyl-coenzyme A and thence into fatty acids and, via the Krebs cycle, into carbon dioxide. The extent to which this occurs will depend on the rate of entrance of pyruvate into the cell and the rate at which it is equilibrated with the endogenously formed pyruvate. The added pyruvate should not depress the metabolism of [1-^{14}C]-glucose to carbon dioxide via the hexose monophosphate pathway but might increase the rate of this reaction if the added pyruvate, by product inhibition, decreased one or more of the steps in glycolysis.

D-2-Deoxyglucose is phosphorylated to D-2-deoxyglucose 6-phosphate and this inhibits the enzyme which converts glucose 6-phosphate into fructose 6-phosphate (Wick, Drury, Nakader & Wolfe, 1957). It apparently does not inhibit glucose 6-phosphate dehydrogenase, for the conversion of [1-^{14}C]glucose into carbon dioxide was not depressed but was increased. This may be attributed to the partial inhibition of glycolysis. The conversion of both C-1 and C-6 of glucose into lipid was greatly depressed by D-2-deoxyglucose as one would expect. The net effect of added pyruvate and D-2-deoxyglucose was to increase the fraction of glucose metabolized by the hexose monophosphate pathway and to decrease the fraction metabolized by glycolysis.

Table 6 summarizes the results of observations on tissues from several species of animals. These emphasize the considerable differences that may

Table 5. *Effects of addition of pyruvate and deoxyglucose on glucose metabolism by human foetal liver*

The values are mean±S.E. expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Incubation medium	No. of expts.	Glucose carbon→CO_2			Glucose carbon→lipid		
		C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
Glucose (11·1 mM)	9	0·68 ±0·070	0·17 ±0·018	0·25 ±0·030	0·17 ±0·016	0·26 ±0·024	1·50 ±0·16
Glucose + pyruvate (10 mM)	8	0·64 ±0·066	0·12 ±0·014	0·19 ±0·028	0·094 ±0·0090	0·22 ±0·021	2·34 ±0·21
Glucose + pyruvate + deoxyglucose (6·1 mM)	4	0·93 ±0·082	0·14 ±0·014	0·15 ±0·023	0·063 ±0·0082	0·18 ±0·019	2·82 ±0·20

Table 6. *Metabolism of* [1-^{14}C]- *and* [6-^{14}C]-*glucose to carbon dioxide and lipid by adult and foetal tissues*

The values are mean±S.E. expressed as μmoles of glucose converted into product after 1 hr./g. fresh wt. of tissue.

Tissue	No. of expts.	Glucose carbon→CO_2			Glucose carbon→lipid		
		C-1	C-6	C-6/C-1	C-1	C-6	C-6/C-1
Liver, rabbit, 25-day foetus	2	0·55	0·10	0·18	0·098	0·102	1·04
Liver, rabbit, newborn	2	0·89	0·15	0·17	—	—	—
Thyroid, dog, adult	11	0·50 ±0·040	0·22 ±0·024	0·43 ±0·048	0·049 ±0·009	0·058 ±0·010	1·18 ±0·28
Thyroid, human, foetal	4	0·24 ±0·031	0·059 ±0·0081	0·246 ±0·028	—	—	—
Thymus, human, foetal	4	0·16 ±0·027	0·017 ±0·0024	0·103 ±0·015	—	—	—
Ovary, rat	5	0·54 ±0·056	0·46 ±0·051	0·85 ±0·12	—	—	—
Adrenal, dog, adult	12	0·26 ±0·033	0·078 ±0·0091	0·30 ±0·036	0·019 ±0·0021	0·029 ±0·0032	1·53 ±0·29
Liver, rat, control	12	1·13 ±0·090	0·091 ±0·014	0·081 ±0·012	0·028 ±0·007	0·050 ±0·009	1·78 ±0·18
Liver, rat, pantothenic acid deficient	8	2·00 ±0·25	0·21 ±0·035	0·106 ±0·014	0·044 ±0·0060	0·140 ±0·033	3·2 ±0·23

exist in the relative importance of the two pathways in tissues from different species of animals. Similar differences were reported recently (Field, Pastan, Herring & Johnson, 1960) in ovary and testis. The ratio of glucose C-6/C-1 metabolized to carbon dioxide reported by Field *et al.*, 0·20 for human ovary and 0·43 for calf ovary, can be compared with the value of 0·85 for rat ovary obtained in these studies. The values were 0·019, 0·17 and 0·45 for the testes of calf, man and rabbit respectively. Not only were the relative rates of conversion of glucose C-1 and C-6 into carbon dioxide different in the tissues of different species, the absolute rate of metabolism per gram of tissue was tenfold greater in calf ovary than in human ovary and fivefold greater in calf testis than in human testis. Although part of the difference might be ascribed to the ages of the donors of the human tissues (the ovaries were those of a woman aged 41 and the testes were those of a man aged 75), it is clear that marked interspecific differences do exist. These marked interspecific differences in the relative rates of glucose metabolized via glycolysis and the hexose monophosphate pathway in a particular kind of tissue suggest that it would be unwise to make extensive generalizations about the possible physiological roles of the phosphogluconate pathway in the intermediary metabolism of a given tissue.

Livers from a series of adult rats maintained on pantothenic acid-deficient diets since their weaning (a total of 12 weeks) were compared with those of litter mates kept on a control diet (Table 6). Although the pantothenic acid-deficient animals were quite ill their livers metabolized glucose at nearly twice the rate of control livers. The conversion of C-1 and C-6 of glucose into both carbon dioxide and lipid was increased but there was no significant difference in the relative rates of metabolism of C-1 and C-6 of glucose. We had supposed that, since both fatty acid metabolism and the tricarboxylic acid cycle require coenzyme A whereas the hexose monophosphate pathway does not, the pantothenic acid-deficient rat might metabolize more of its glucose via the latter pathway. This expectation was not realized and it would appear that rats with all the signs of severe pantothenic acid deficiency still have enough coenzyme A in their tissues for these enzymic processes.

DISCUSSION

In all of the tissues examined there was no demonstrable change in the relative rates of metabolism via these two alternative pathways of carbohydrate metabolism over the period of foetal development measured, roughly 8–25 weeks' gestational age. Each tissue has developed, sometime before the eighth week, a pattern of carbohydrate metabolism which is characteristic for it. There is no evidence as to when the hexose monophosphate pathway may first appear in these foetal tissues. In sea-urchin eggs undergoing cleavage and gastrulation, the hexose monophosphate pathway appears to be the major one of glucose oxidation, whereas some 24 hr. later glycolysis and the Krebs cycle have become predominant. This was found by Krahl (1956) and our own experiments with sea-urchin embryos (Villee, Loring & Wellington, 1957) have confirmed and extended his observations. It is possible that an analogous situation exists in the human egg during the early blastocyst stage.

The observation that these pathways of carbohydrate metabolism are relatively unchanging throughout foetal life and resemble the adult condition is in some contrast with a number of the reactions of amino acid metabolism and of glucuronic acid metabolism. As Kretchmer (1959) has shown, there are marked changes in the rates of metabolism of tyrosine, tryptophan and certain other amino acids in the late foetal and early newborn periods. Lathe (1959) and Brown, Zuelzer & Bollet (1958) have demonstrated that the enzymes involved in the synthesis of uridine diphosphoglucuronic acid undergo development late in foetal life.

The effects on carbohydrate metabolism observed when pyruvate and 2-deoxyglucose were added to the medium were to be expected on the basis of our present knowledge of intermediary metabolism. They lend confidence to this method of measuring and interpreting alternative pathways of carbohydrate metabolism in tissue slices.

SUMMARY

1. Tissue slices were incubated with [1-^{14}C]-glucose and [6-^{14}C]glucose to evaluate the relative rates of glycolysis and the hexose monophosphate oxidative pathway in a variety of foetal and adult tissues.

2. The effects of anaerobiosis and of the addition of unlabelled pyruvate, D-2-deoxyglucose, ascorbate, and di- and tri-phosphopyridine nucleotide were tested.

3. There was no significant change in the relative rates of metabolism via these two alternative pathways of carbohydrate metabolism in any of the tissues over the period of foetal development from 8 to 25 weeks' gestational age.

4. The activity of the hexose monophosphate pathway in different tissues ranged from the foetal cerebral cortex, in which it is essentially zero, to the adrenal cortex, in which it accounts for most of the glucose metabolized. Comparative studies,

however, revealed wide differences in the relative activities of the two pathways in comparable tissues of different species.

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A Study of the Citric Acid Cycle in Certain Tumour Tissues

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At present there are two schools of thought about the respiration of cancer cells. Whereas both agree that tumour tissues exhibit high anaerobic and aerobic glycolysis with an excessive production of lactic acid (Cori & Cori, 1925; Warburg, 1926, 1956), they differ as to the extent of the operation of the citric acid cycle in these tissues (Weinhouse, 1955; Weinhouse, Warburg & Burk, 1956; Warburg, 1956). In these studies the activity of certain segments of the cycle has been determined by oxygen consumption and carbon dioxide production procedures (Warburg, 1926; Busch & Potter, 1952; Busch & Baltrush, 1954; Busch, 1955; Weinhouse, 1955; Warburg, 1956; Ibsen, Coe & McKee, 1958), by measuring the glycolytic and oxidative enzymes (Sibley & Lehninger, 1949; LePage, 1950; Meister, 1950; Potter, 1951; Weinhouse, 1951; Wenner, Spirtes & Weinhouse, 1952; Beck, 1958), and the cofactors of the electron-transport system (Dubois & Potter, 1942; Wenner & Weinhouse, 1953; Greenstein, Werne, Eschenbrenner & Leuthardt, 1944), or by following the utilization of the various intermediates of the Embden–Meyerhof scheme and the tricarboxylic acid cycle (Pardee & Potter, 1949; Elliott & Kalnitsky, 1950; Olson, 1951; Wenner & Weinhouse, 1952; Heidelberger, 1953; Busch & Baltrush, 1954). However, none of these investigators has utilized the simultaneous determination of the amounts and radioactivities of all the members of the citric acid cycle *in vitro* in the presence and absence of certain added labelled substrates and blocking agents. Such a procedure could reflect the activity of the cycle if an excess of substrate is present despite the fact that the individual cycle acids allegedly serve a 'catalytic' function. There are examples of variations in the amounts of the individual cycle acids under differing experimental conditions, for example, after the addition of specific blocking agents. The present study was designed to employ such a direct approach. The tumour and normal tissue preparations were incubated with [^{14}C]acetate,

[^{14}C]succinate or non-labelled malate and the amounts and activities of the individual cycle acids were determined as described by Dajani & Orten (1958). The results conclusively demonstrated a quantitatively reduced activity of the tricarboxylic acid cycle in the tumours as compared with the normal tissues studied under the conditions of the experiments.

EXPERIMENTAL

Materials

DPN, ATP, succinic acid and L-malic acid, all purified grade, were purchased from the California Foundation for Biochemical Research, Los Angeles, Calif. [1-^{14}C]Acetate and [1.^{14}C]succinate were obtained from Volk Radiochemical Co., Chicago, Ill. Penicillin G potassium was a product of Lederle Laboratories, New York; the dihydrostreptomycin was produced by Pfizer and Co., New York. Triethyltin acetate was kindly supplied by Dr K. E. Moore, University of Michigan, and by the Metal and Thermite Corp., New York City.

Three types of tumour tissues were used: Ehrlich mouse-ascites-tumour cells, and Walker and LC-18 carcinoma from adult rats. The Walker carcinoma was originally a human adenocarcinoma of the adrenals and the LC-18 carcinoma was originally a human hepatoma. Both had undergone repeated transfers in Fisher strain white rats and were implanted and grown subcutaneously. The normal tissues used for comparison were muscle, liver and isolated liver cells from Fisher strain adult rats and nucleated erythrocytes from Plymouth Rock chickens.

Procedure

Twenty per cent homogenates of pooled tumour and non-tumour tissues from 8–10 rats were prepared at 5° in 50 ml. of Krebs–Ringer phosphate buffer (pH 7·4) with the use of a Potter–Elvehjem glass homogenizer. To the homogenates were added the following substances, together with the various substrates used, dissolved in 5 ml. of Krebs–Ringer phosphate: L-malic acid, 0·05 mg.; ATP, 8·0 mg.; DPN, 2·0 mg.; monofluoroacetic acid (where used), 0·5 mg.; sodium malonate (where used), 75·0 mg.; penicillin, 1–2 mg.; streptomycin, 2–4 mg. Sodium fluoride (0·01 M final concentration) was added to the Krebs–Ringer phosphate used in homogenization in order to prevent loss of ATP and DPN by adenosine triphosphatase and diphosphopyridine nucleotidase (NAD nucleosidase) respectively. In an additional series of experiments triethyltin acetate, a recently introduced powerful and complete adenosine-triphosphatase inhibitor (Dr K. E. Moore, personal communication), was added to the fluid mixture used in the homogenization of tissues in the recommended final concentration (1 μM). The supplementary DPN, ATP, as well as triethyltin acetate, were added as a further precautionary measure because there is some evidence that homogenized tissues, particularly certain tumours, exhibit increased activity of adenosine triphosphatase and probably diphosphopyridine nucleotidase. The amounts of the substrates used are given as subheadings to the appropriate Tables. The addition of these substrates to the tissue preparations followed the procedure described elsewhere (Dajani & Orten, 1958). The mixtures were incubated at 37–38° in O_2+CO_2 (95:5) for 30 min. and 1, 4, 18 or 24 hr., with constant gentle shaking (250 oscillations/min.). Since incubation for the shorter intervals did not result in an accumulation of the cycle acids to significantly measurable quantities in the presence of inhibitors, the 4 hr. time interval was chosen for incubation in all subsequent experiments. Although a longer period, 18 hr., gave higher values, the picture obtained was similar and the shorter period was adopted. In all of these trial studies excess of substrate was always detected at the end of the experiment. However, after incubation for 24 hr. no substrate remained, and the levels of the cycle acids were diminished. It should be emphasized at this point that all the experimental conditions adopted were also found to support vigorous Krebs-cycle activity in nucleated erythrocytes (Dajani & Orten, 1958). As a further check, the pH of the mixtures was determined at the end of the 4 hr. period. No significant change had occurred. At the end of the incubation period, the enzymic reactions were stopped by freezing, and the extraction and determination of the citric acid-cycle acids and their ^{14}C-activities followed the procedure that has already been reported (Dajani & Orten, 1958). The citric acid-cycle acids were extracted with acid–acetone and the amounts and ^{14}C-activities of the individual cycle acids were quantitatively determined after separation by silica-gel-column chromatography by a procedure developed in this Laboratory and described in detail by Dajani & Orten (1958). Essentially the procedure involved the differential elution of the cycle acids from the silica-gel column by automatically increasing concentrations of *tert.*-amyl alcohol in chloroform. The eluted acids were collected in an automatic fraction collector and then titrated with standard sodium hydroxide by using a Rehburg microburette. The titrated fractions were then transferred to planchets, evaporated to dryness and the ^{14}C-activities determined in a gas-flow counter. The flow of effluent through the chromatographic column was slowed by the excessive amounts of lipid in the extracts of tumour tissue, resulting in a poor separation of the cycle acids, so the procedure was modified to remove lipid. For this purpose, the aqueous concentrate of the acid–acetone extract was shaken in a separating funnel with ether (10 ml.). After 15 min., the ether layer was transferred to aluminum planchets and evaporated to dryness, and the radioactivity was determined. The aqueous layer was used for determination of the cycle acids (Dajani & Orten, 1958). No significant loss of cycle acids by this treatment was found in recovery studies.

Preparation of cell suspensions. Ehrlich mouse-ascites-carcinoma fluid containing the tumour cells was aspirated from the peritoneal cavity of 20–25 ether-anaesthetized mice. The fluid was pooled and the free cells were obtained by differential centrifuging at 200*g* for 5 min. and then at 3000*g* for 15 min. The cells were washed twice with Krebs–Ringer phosphate and suspended in 50 ml. of Krebs–Ringer phosphate (pH 7·4), or Krebs–Ringer phosphate containing 20% of clear ascites fluid, to make a 20% cell suspension. Hepatic cells from normal rats were isolated by the procedure of Branster & Morton (1957). Nucleated erythrocytes were prepared as described by Dajani & Orten (1958). These cells were suspended in 50 ml. of Krebs–Ringer phosphate, to give a 20% suspension. The rest of the procedure was the same as that given above.

RESULTS

Experiments represented in Tables 1 and 2 on normal and tumour tissues permit a comparison of the utilization of [1-^{14}C]acetate by way of the Krebs cycle in these tissues. The amounts of acids listed in these and subsequent Tables are increases over controls (no substrate added). It is evident that the concentrations and ^{14}C-activities of the citric acid-cycle intermediates are lower in the tumour tissues than in normal liver or muscle. Also, there is more incorporation of ^{14}C into the cycle acids in LC-18 carcinoma tissue than in Walker carcinoma.

In the studies represented in Tables 1 and 2, it may be recalled that fluoride was added to the homogenates of the tissues to inhibit adenosine triphosphatase and diphosphopyridine nucleotidase. Since it appears possible that fluoride may not have completely inhibited these phosphatases when present at the elevated levels claimed in tumour tissues, triethyltin acetate was employed as an adenosine-triphosphatase inhibitor in place of fluoride. The results, given in Table 3, confirm those found when fluoride was used and thus indicate that the lower activity of the cycle, in LC-18 tumour tissues at least, is not due to a loss or destruction of ATP.

Table 1. *Average concentrations (μmoles/100 g.) of Krebs-cycle acids in tissues incubated with* [1-^{14}C]*acetate*

Values are net syntheses above controls and are averages of three experiments ± average deviations, with different batches of pooled tissues. The substrate contained: 0·45 mg. of [1-^{14}C]acetate (4·11 mc/m-mole); 4·0 mg. of sodium acetate trihydrate. These were dissolved in the incubation mixture, which contained in each 50 ml. of Krebs–Ringer phosphate buffer (pH 7·4): L-malic acid, 0·05 mg.; ATP, 8·0 mg.; DPN, 2·0 mg.; NaF, 21·0 mg.; penicillin, 2 mg.; dihydrostreptomycin, 4 mg.; monofluoroacetic acid (where used), 0·5 mg. No substrate was added to the controls.

Acid	Control	Acetate	Acetate + fluoroacetate	Control	Acetate	Acetate + fluoroacetate
		Liver			Muscle	
Citric	1·1±0·02	3·0±0·06	2·0±0·02	1·0±0·01	3·3±0·03	4·6±0·08
Aconitic (*cis*+*trans*)	0·4±0·01	0·6±0·02	0·9±0·01	0·3±0·01	0·5±0·01	0·8±0·02
*iso*Citric	0·5±0·01	0·6±0·01	0·8±0·01	0·4±0·02	0·7±0·02	0·8±0·01
α-Oxoglutaric	1·3±0·03	3·8±0·08	2·2±0·02	0·9±0·01	2·4±0·06	2·1±0·02
Succinic	1·7±0·07	4·4±0·07	3·8±0·06	1·5±0·03	4·2±0·07	3·9±0·06
Fumaric	1·5±0·06	3·8±0·05	3·6±0·05	1·4±0·02	3·9±0·05	3·7±0·08
L-Malic	1·3±0·03	3·5±0·06	3·3±0·03	1·3±0·04	3·6±0·05	3·5±0·07
Oxaloacetic + acetoacetic	0·2±0·01	1·0±0·02	6·2±0·07	0·3±0·01	0·8±0·02	5·9±0·05
		Walker carcinoma			LC-18 carcinoma	
Citric	0·5±0·01	0·7±0·02	1·5±0·03	0·5±0·07	0·6±0·03	1·7±0·06
Aconitic (*cis*+*trans*)	0·2±0·03	0·5±0·04	0·9±0·02	0·3±0·02	0·5±0·04	1·1±0·03
*iso*Citric	0·3±0·03	0·5±0·03	0·9±0·01	0·3±0·04	0·6±0·02	1·1±0·01
α-Oxoglutaric	0·4±0·03	1·5±0·06	1·4±0·06	0·3±0·03	1·7±0·03	1·5±0·03
Succinic	0·2±0·01	1·4±0·04	1·0±0·04	0·6±0·02	2·1±0·05	1·8±0·08
Fumaric	0·3±0·04	1·4±0·03	1·0±0·03	1·5±0·06	2·4±0·05	1·9±0·07
L-Malic	0·3±0·03	1·4±0·02	0·9±0·04	0·4±0·04	1·2±0·04	1·2±0·04
Oxaloacetic + acetoacetic	0·4±0·02	1·3±0·02	2·9±0·06	0·3±0·03	1·3±0·03	3·8±0·08

Table 2. *Average* ^{14}C *counts* (10^{-3} × *counts/min./100 g.) of Krebs-cycle acids in tissues incubated with* [1-^{14}C]*acetate*

Values are averages of three experiments, with different batches of pooled tissues. The substrate and composition of the incubation mixture are given in Table 1.

	Liver		Muscle		Walker carcinoma		LC-18 carcinoma	
Acid	Acetate	Acetate + fluoro-acetate	Acetate	Acetate + fluoro-acetate	Acetate	Acetate + fluoro-acetate	Acetate	Acetate + fluoro-acetate
Citric	110·0	105·0	125·0	203·0	7·4	8·7	7·9	9·5
Aconitic (*cis*+*trans*)	30·0	31·0	38·0	30·0	3·2	2·7	3·4	2·8
*iso*Citric	38·0	32·0	36·0	34·0	3·2	2·6	3·3	2·7
α-Oxoglutaric	95·0	61·0	68·0	59·0	4·3	4·0	4·8	4·2
Succinic	125·0	96·0	122·0	115·0	2·2	2·1	2·9	2·4
Fumaric	104·0	100·0	113·0	110·0	2·1	2·0	3·2	2·5
L-Malic	92·0	87·0	109·0	107·0	2·0	1·8	3·6	2·0
Oxaloacetic + acetoacetic	27·0	297·0	22·0	168·0	4·5	8·4	4·6	12·0

Table 3. *Average concentration and ^{14}C-activity of Krebs-cycle acids in tissue homogenates incubated with [1-^{14}C]acetate in the presence of triethyltin acetate*

Values are net syntheses above controls and are averages of two experiments with different batches of pooled tissues. The compositions of the substrate and incubation mixture are given in Table 1, with the exception that triethyltin acetate at a final concentration of 1 μM replaced NaF as an adenosine-triphosphatase inhibitor.

	Liver		LC-18 carcinoma	
Acid	(μmoles/100 g.)	(10^{-3} × Counts/min./100 g.)	(μmoles/100 g.)	(10^{-3} × Counts/min./100 g.)
Citric	3·2±0·02	112·0	0·7±0·02	8·1
Aconitic (*cis*+*trans*)	0·7±0·02	33·0	0·4±0·03	3·1
*iso*Citric	0·6±0·03	35·0	0·5±0·03	3·3
α-Oxoglutaric	4·1±0·05	100·0	1·8±0·04	4·0
Succinic	4·3±0·02	122·0	2·0±0·05	3·0
Fumaric	4·0±0·06	108·0	2·3±0·04	3·3
L-Malic	3·6±0·05	94·0	1·3±0·07	3·8
Oxaloacetic+acetoacetic	1·2±0·03	30·0	1·4±0·03	4·9

Table 4. *Cycle acids in ascites-tumour cells incubated with [1-^{14}C]acetate+20 % of ascites fluid*

Values are net syntheses above controls and are averages of three experiments, ±average deviations, with different batches of pooled tissues. The compositions of the substrate and the incubation mixture are given in Table 1. Where used, 75·0 mg. of sodium malonate was added to the incubation mixture. No substrate was added to the controls. Blank values indicate amounts too small to be measured accurately.

	μmoles/100 g. of packed cells				10^{-3} Counts/min./100 g. of packed cells		
Acid	Control	Acetate	Fluoro-acetate added	Malonate added	Acetate	Fluoro-acetate added	Malonate added
Citric	—	1·5±0·03	2·3±0·05	2·1±0·06	4·3	6·4	6·0
Aconitic (*cis*+*trans*)	—	—	—	—	1·4	1·3	1·6
*iso*Citric	—	—	—	—	1·4	1·2	1·6
α-Oxoglutaric	—	1·3±0·02	1·0±0·03	1·2±0·02	3·3	2·9	3·4
Succinic	0·9±0·03	2·0±0·04	1·8±0·05	2·4±0·03	5·4	5·3	6·9
Fumaric	0·7±0·02	1·9±0·05	1·8±0·07	1·1±0·01	5·1	4·9	4·3
L-Malic	—	1·6±0·04	1·5±0·03	1·5±0·05	4·2	3·9	3·9
Oxaloacetic+acetoacetic	—	2·7±0·06	3·3±0·07	2·4±0·08	7·5	9·4	6·9

The amounts of lactic acid and pyruvic acid in the different tissues were also determined together with the cycle acids in all experiments. The concentrations of these two acids were invariably greater in all tumour tissues studied than in the normal tissues. This observation is in agreement with that of other investigators (Olsen, 1951; Heidelberger, 1953; Warburg, 1956; Weinhouse *et al.* 1956).

Because the Walker and LC-18 tumours are a composite tissue and have a varying cancer-cell content, their use could, undoubtedly, lead to uncertainty in interpretation. It was thought advisable, therefore, to carry out further experiments on Ehrlich mouse-ascites-tumour cells, which are a nearly pure culture of intact cancer cells. Moreover, the utilization of unruptured ascites cells minimizes the uncertainty of ATP loss resulting from homogenization. At first, suspensions of these cells in Krebs–Ringer phosphate alone were used. The results of three experiments on different preparations of ascites cells indicated that acetate was incorporated into the various citric acid-cycle acids to a lesser degree than that in the normal tissues. Most of the substrate appeared to have been diverted mainly to the formation of acetoacetate and fatty acids. Indeed, a greater portion of the ^{14}C-activity resided in the ether extract mentioned above. In further experiments, in order to provide more nearly physiological conditions for incubation, 20 % of ascites fluid was incorporated into the incubation medium, replacing an equivalent amount of Krebs–Ringer phosphate. The use of ascites fluid in the incubation medium has been found advantageous by others (Kun, Talalay & Williams-Ashman, 1951; Warburg, 1956). Indeed, in the present study, the inclusion of ascites fluid in the incubation mixture employed in this and the subsequent experiments increased the concentrations and activities of the citric acid-cycle acids over those obtained with Krebs–Ringer phosphate alone. As is evident from the data presented in Table 4, however, the activity of the cycle is less than that of the normal tissues studied (Tables 1 and 2).

Since high concentrations of DPN have been reported to increase the oxygen uptake of hepatoma and liver mitochondria (Wenner & Weinhouse, 1952), the effect of the two concentrations of this cofactor (2 or 5 mg.) on the utilization of acetate by the ascites-tumour cells was studied. In spite of the fact that the addition of DPN did not seem to raise the concentrations and activities of the Krebs-cycle intermediates to any appreciable extent, its inclusion in the incubation mixture, as stated earlier, was continued simply as a precautionary measure.

In addition to the effect of fluoroacetate upon the activity of the Krebs cycle, malonate, a specific inhibitor of succinic dehydrogenase (Pardee & Potter, 1949), was also studied. Its use as a blocking agent should result in the accumulation of succinate. The results in Table 4 show that, although there was an accumulation of succinate, this was small. As with fluoroacetate inhibition, this observation points to a reduced activity of the cycle in these cells. This is not in complete disagreement with the experiments *in vivo* of other investigators (Busch & Potter, 1952), who demonstrated that several tissues, including five tumours, accumulated succinate in the presence of malonate. Their data were interpreted to mean only that the Krebs cycle is functioning in these tissues, and do not point to the extent of the cycle activity. Despite this finding, however, these authors cautioned against drawing definite conclusions based on their data alone, since succinate may have arisen from sources other than the citric acid-cycle intermediates, e.g. glutamic acid.

The results given thus far strengthen the thesis that the citric acid cycle is operating at a lower level in the tumour tissues studied than in normal tissues. This could be due to a block(s) of an unknown nature either within or outside the cycle, hindering acetate from entering into the cycle. In order to obtain further evidence on the former point, two of the intermediates of the cycle itself were employed as substrates, in addition to acetate.

In one series, non-labelled L-malic acid mixed with labelled acetate was incubated with the tumour-cell suspension. In another, radioactive succinate mixed with non-labelled acetate was used. The results of these experiments are given in Tables 5 and 6 respectively. Again, the data clearly demonstrated that these two intermediates were not utilized by the cycle enzymes better than was acetate alone (Table 4). This was expected, at least with respect to succinate, since the succinic-dehydrogenase activity in tumour tissue is believed to be low (Schneider & Potter, 1943). Further, the occurrence of a weak electron-transport chain reported by Schneider & Potter (1943) and others (Greenstein *et al.* 1944) could also be a contributing factor. The use of fluoroacetate and malonate as inhibitors, also, did not significantly alter the concentrations of citrate or succinate respectively. This finding is similar to that obtained above when acetate alone was used as a substrate (Table 4). If there were an impairment in the pathway between acetate and citrate, one of two possibilities, or both, could be a contributing factor(s). Either the activating-enzyme system of acetate which brings about the formation of acetyl-CoA or the condensing enzyme might not be functioning normally. Other studies, however, appear to rule out the latter possibility (Wenner *et al.* 1952). This leaves the former as a possible alternative mechanism. One could speculate, therefore, that acetyl-CoA may not have been biosynthesized in sufficient quantities or that its production was at such a slow rate that it could not keep pace with the turnover by the condensing enzyme. To explore this point, the systems of the experiments that follow were fortified with 5 mg. of CoA to test its effect on the utilization of acetate or succinate by

Table 5. *Cycle acids in ascites-tumour cells incubated with substrate containing* 1·5 *mg. of* L-*malic acid*

Values are net syntheses above controls and are averages of three experiments, ±average deviations, with different preparations of ascites cells. The compositions of the substrate and incubation mixture are given in Tables 1 and 4. Blank values indicate amounts too small to be measured accurately.

	μmoles/100 g. of packed cells				10^{-3} × Counts/min./100 g. of packed cells		
Acid	Control	Acetate + malate	Fluoro-acetate added	Malonate added	Acetate + malate	Fluoro-acetate added	Malonate added
Citric	—	1·4±0·02	2·3±0·07	2·3±0·07	4·5	6·5	6·1
Aconitic (*cis* + *trans*)	—	—	—	—	1·3	1·3	1·4
*iso*Citric	—	—	—	—	1·4	1·2	1·4
α-Oxoglutaric	—	1·3±0·03	1·2±0·08	1·5±0·04	3·4	3·2	3·3
Succinic	0·8±0·03	2·3±0·04	2·0±0·1	2·5±0·08	5·3	5·3	6·3
Fumaric	0·7±0·02	2·2±0·05	1·9±0·04	1·7±0·09	5·0	4·9	4·4
L-Malic	—	1·9±0·04	1·7±0·05	2·0±0·06	4·5	4·1	4·1
Oxaloacetic + acetoacetic	—	3·0±0·08	3·4±0·06	3·3±0·06	7·8	9·7	6·9

Table 6. *Cycle acids in ascites-tumour cells incubated with* [1-^{14}C]*succinic acid*

Values are net syntheses above controls and are averages of two experiments, ±average deviations, with different preparations of ascites cells. The substrate contained: succinic acid, 5·0 mg.; [1-^{14}C]succinic acid, 1·5 mg. (activity 1·3 mc/m-mole); sodium acetate trihydrate, 2·0 mg.; in 50 ml. of Krebs–Ringer phosphate. The composition of the incubation mixture is given in Table 1. Blank values indicate amounts too small to be measured accurately. The high counts for succinic acid are due primarily to non-utilized [1-^{14}C]succinic acid.

	μmoles/100 g. of packed cells			10^{-3} × Counts/min./100 g of packed cells	
Acid	Control	Succinate	Succinate + fluoroacetate	Succinate	Succinate + fluoroacetate
Citric	—	1·3±0·03	1·4±0·02	11·5	12·0
Aconitic (*cis* + *trans*)	—	—	—	5·8	4·7
*iso*Citric	—	—	—	5·3	4·0
α-Oxoglutaric	—	0·8±0·01	0·7±0·03	7·5	7·8
Succinic	0·9±0·02	20·8±0·08	21·0±0·04	361·5	395·0
Fumaric	0·8±0·02	1·7±0·04	1·6±0·06	18·8	18·0
L-Malic	—	1·6±0·07	1·7±0·05	17·4	16·8
Oxaloacetic + acetoacetic	—	1·1±0·03	1·2±0·06	14·0	14·5

Table 7. *Cycle acids in ascites-tumour cells incubated with substrate containing acetyl-coenzyme A*

Values are net syntheses above controls and are from one experiment. The compositions of the substrate and incubation mixture are given in Table 1, with the exception that 2 μmoles of acetyl-CoA were added. No substrate was added to the controls. Blank values indicate amounts too small to be measured accurately.

	μmoles/100 g. of packed cells		10^{-3} × Counts/min./100 g. of packed cells.
Acid	Control	Acetate + acetyl-CoA + fluoroacetate added	Acetate + acetyl-CoA + fluoroacetate added
Citric	—	2·3	6·4
Aconitic (*cis* + *trans*)	—	—	1·3
*iso*Citric	—	—	1·2
α-Oxoglutaric	—	1·2	2·9
Succinic	0·9	2·0	5·3
Fumaric	0·7	1·9	4·9
L-Malic	—	1·5	3·9
Oxaloacetic + acetoacetic	—	3·4	9·3

way of the citric acid cycle in ascites cells. If CoA is lacking or not present in adequate amounts, its addition to the incubation medium would be expected to raise the concentrations of the cycle members, provided that the other cofactors and the activating enzyme are not only present but are also functioning normally. It is assumed, of course, that this would be the case if the cycle itself is also operating at a normal level. When it was found that CoA failed to increase the concentrations of the cycle acids significantly under the experimental conditions, a further investigation with preformed acetyl-CoA was pursued (Table 7) in an effort to determine whether or not the activating system itself might be defective. Contrary to expectation, no increase in citrate in the presence of fluoroacetate was observed (cf. Table 4), a finding that can be considered as indirect evidence against an impairment of the acetate-activating system. The only increase under these conditions was in the oxaloacetate–acetoacetate fraction. This observation will be discussed later.

To assess still further the operation of the citric acid cycle in ascites-tumour cells, the activity of the cycle was compared under identical conditions with that in isolated liver cells and in nucleated erythrocytes (also suspensions of intact cells). The data (Table 8) show that the quantities and activities of the cycle members biosynthesized in hepatic cells and in nucleated erythrocytes are several times greater than those found in ascites-tumour cells. Particularly striking is the accumulation of citrate in nucleated erythrocytes when fluoroacetate is added. Likewise, when malonate is added to hepatic cells, there is a marked accumulation of succinate.

Table 8. *Comparison of cycle acids in ascites-tumour cells, isolated hepatic cells and nucleated erythrocytes incubated with the same substrate*

Values are net syntheses above controls and are averages of three experiments, ± average deviations, with different preparations of pooled cells. The compositions of the substrate and incubation mixture are given in Tables 1 and 4. Blank values indicate amounts too small to be measured accurately. The values for nucleated erythrocytes are taken from Dajani & Orten (1958).

Acid	μmoles/100 g. of packed cells			10^{-3} × counts/min./100 g. of packed cells		
	Acetate	Fluoroacetate added	Malonate added	Acetate	Fluoroacetate added	Malonate added
			Ascites cells			
Citric	1·5±0·03	2·3±0·05	2·1±0·06	4·3	6·4	6·0
Aconitic (*cis*+*trans*)	—	—	—	1·4	1·3	1·6
*iso*Citric	—	—	—	1·4	1·2	1·6
α-Oxoglutaric	1·3±0·02	1·0±0·03	1·2±0·02	3·3	2·9	3·4
Succinic	2·0±0·04	1·8±0·05	2·4±0·03	5·4	5·3	6·9
Fumaric	1·9±0·05	1·8±0·07	1·1±0·01	5·1	4·9	4·3
L-Malic	1·6±0·04	1·5±0·03	1·5±0·05	4·2	3·9	3·9
Oxaloacetic + acetoacetic	2·7±0·06	3·3±0·07	2·4±0·08	7·5	9·4	6·9
			Isolated hepatic cells			
Citric	3·0±0·02	1·9±0·03	1·9±0·05	113·0	112·0	110·5
Aconitic (*cis*+*trans*)	0·7±0·01	0·6±0·04	0·6±0·04	30·5	29·5	32·5
*iso*Citric	0·8±0·02	0·5±0·02	0·5±0·02	38·0	34·0	35·0
α-Oxoglutaric	4·2±0·04	2·6±0·03	4·5±0·05	97·0	63·0	102·0
Succinic	4·3±0·03	3·9±0·05	6·7±0·06	128·0	99·0	165·5
Fumaric	3·8±0·06	3·6±0·06	2·1±0·03	107·0	101·0	75·8
L-Malic	3·3±0·06	3·2±0·04	2·2±0·04	93·0	88·0	66·0
Oxaloacetic + acetoacetic	1·2±0·02	5·8±0·05	3·5±0·07	29·0	212·0	97·0
			Nucleated erythrocytes			
Citric	28·5	49·5		1380·0	2310·0	
Aconitic (*cis*+*trans*)	1·6	1·1		67·2	48·5	
*iso*Citric	1·6	1·1		68·8	47·9	
α-Oxoglutaric	16·0	3·6		432·0	95·5	
Succinic	49·7	48·5		1650·0	1740·0	
Fumaric	51·0	49·5		1440·0	1390·0	
L-Malic	39·5	39·0		1220·0	1080·0	
Oxaloacetic + acetoacetic	28·0	135·0		785·0	3780·0	

DISCUSSION

It appears from work in other laboratories (see references cited in introduction), with manometric, enzymic and isotopic techniques, that segments of the citric acid cycle proceed, to some extent at least, in tumour tissue. On the other hand, there is also evidence which indicates that the activity of the cycle is of a lower order than that of many normal tissues. The results presented in the present paper, however, indicate clearly that the activity of the citric acid cycle in the tumour tissues studied is of a lower order than that in the normal control tissues. This is based on the interpretation that the concentrations and total ^{14}C-activities of the intermediates in the cycle reflect the amount of material passing through it. Another possible interpretation, on the other hand, is that the lower concentrations of the acids in the tumour tissues could mean that the cycle is as active or even more active in these tissues, provided that the enzyme levels are higher. However, measurement of the levels of certain enzymes of the citric acid cycle and some other related enzymes, have shown that they are either comparable or lower, but never higher, in tumour tissues. The enzymes measured include cytochrome oxidase (Greenstein *et al.* 1944), succinic dehydrogenase and cytochrome oxidase (Schneider & Potter, 1943), aconitase (Weinhouse, 1951), malic dehydrogenase (Potter, 1946), condensing enzyme (Potter & Busch, 1950), and lactic, α-oxoglutaric, malic and *iso*citric dehydrogenases, fumarase, oxaloacetic carboxylase, condensing enzyme and aconitase (Wenner *et al.* 1952). Further, an equal or higher activity of the cycle in tumour tissues would require a higher rate of acetate utilization when acetate was used as the substrate. Actually, acetate utilization has been found by several investigators to be much less in tumour than in the normal tissues that they have studied. Pardee, Heidelberger & Potter (1950) measured the oxidation of [1-^{14}C]acetate to $^{14}CO_2$ in slices and homogenates of tumour and non-tumour tissues and found that less than one-

twenty-fifth as much $^{14}CO_2$ was produced by tumour as by kidney. Similar results were later obtained by Busch & Baltrush (1954). Another line of evidence for the occurrence of a reduced activity of the Krebs cycle was provided by Kit & Greenberg (1951), who were able to show that acetate was oxidized to carbon dioxide in Gardner lymphosarcoma to about one-third the extent of that in spleen, one of the normal tissues of relatively low oxidizing activity. Also, citrate accumulation was almost of the same magnitude in the presence of fluoroacetate as in its absence. Weinhouse (1951), on the other hand, has shown that $^{14}CO_2$ is formed in approximately equal quantities from various substrates, including acetate, by tumour and normal-tissue slices. Further, when [2-^{14}C]pyruvate is used as a substrate, $^{14}CO_2$ production in hepatoma was nearly the same as that produced in liver (Olson, 1951). Thus most of the observations *in vitro* cited above indicate a lower activity of the cycle in the tumour tissues studied, as was found by a different procedure in the present study.

Further evidence of a diminished activity of segments of the citric acid cycle have been furnished by several experiments *in vivo*. Potter & Busch (1950) determined the citric acid content of tissues of fluoroacetate-treated rats and found it to accumulate in most tissues with the exception of liver, testis and tumour. The inability of the liver, which is known to exhibit high citric acid-cycle activity, to accumulate citrate was explained on the basis of the occurrence of another pathway for acetate and pyruvate utilization, namely acetoacetate formation. The absence of citrate accumulation in tumour was considered as 'strong evidence that the ability of tumour tissues to form citrate is very low in comparison with a wide variety of normal tissues'. Other experiments on transplanted rat tumour *in vivo* also showed either no significant acetate or pyruvate oxidation (Busch & Baltrush, 1954; Busch, 1955), or, with the uptake of glutamate or succinate, activity of only a portion of the citric acid cycle (Nyhan & Busch, 1958).

Recently Busch, Davis & Olle (1957) have demonstrated that only 'under optimal conditions (i.e. excess of a variety of substrates of the citric acid cycle, pH 7·4, and an atmosphere of 100 per cent oxygen) is the capacity of transplantable rat tumour for production of citrate essentially the same as that of a variety of non-tumour tissues'. Even under such unphysiological conditions as these, their data show that citrate accumulation was significantly greater in the normal tissues than in any of the tumours investigated.

The present study provides a more complete and direct demonstration of a low activity of the entire Krebs cycle in certain tumours. It is evident that, with the exception of the oxaloacetate fraction, which contains an appreciable quantity of acetoacetate, the amounts and activities (Tables 1 and 2) of all the cycle intermediates in liver or muscle were always greater than in the two tumour tissues. The experiments with fluoroacetate furnished further support for this observation. The apparent increase in the oxaloacetate fraction would be expected from the effect of fluoroacetate inhibition of the citric acid cycle and the diversion of a considerable portion of acetate towards fatty acid biosynthesis (Crandall, Gurin & Wilson, 1947).

The studies of Ehrlich ascites cells gave even more convincing evidence about the status of the cycle in tumours. Here again, the quantities and activities of the cycle acids (Table 4) are decidedly less than those in isolated liver cells or nucleated erythrocytes (Table 8). Further, the values are less than those for liver or muscle (Tables 1 and 2). These differences are also apparent if specific activities, which reflect rate of turnover and hence the extent of the activity of the cycle enzymes, are calculated and then compared. Indeed, the specific activities of the individual intermediates in tumours are definitely much less than their counterparts in the normal tissues.

Although the foregoing observations tend to support the view that the Krebs cycle is of low activity in tumours, they have left undecided the question of whether or not a block(s) exists outside the cycle which could hinder the normal entrance of acetate into it. One could assume either an enzyme or a cofactor, or both, to be the limiting factor(s). As a matter of fact, several cofactors, such as DPN, ATP and CoA, are known to be involved in these reactions and would retard the rate of citrate biosynthesis if they were not present in sufficient quantities. Also, two enzyme systems are recognized as being concerned with the reactions that lead to citrate formation from acetate, namely the activating and condensing enzymes. The latter, however, is not believed to exist in tumours in levels comparable with those in non-tumours (Wenner *et al.* 1952). Because DPN and ATP were used in all experiments, including the control, only the CoA was tested for its effect on the utilization of acetate or succinate by way of the citric acid cycle in the ascites cells. The present results make it clear that the cycle components did not increase any more in the presence of CoA than in its absence. This would suggest that CoA either does not penetrate the cell readily or that tumours are less able to activate acetate than many normal tissues. Although no attempt was made to investigate the permeability of the ascites cell to CoA in the present investigation, the formation of acetyl-CoA was studied by indirect means. If acetate

were not normally metabolized via the citric acid cycle because of a lack or a subnormal activity of the activating enzyme, it would be reasonable to assume that adding preformed acetyl-CoA would increase the output of citrate in the presence of fluoroacetate. Acetyl-CoA did not produce any such increase in the citrate concentration (Table 8). It is, of course, possible that acetyl-CoA may also not have penetrated the cell or that it may have been deacylated. The simplest explanation of the inactivity of acetyl-CoA is, of course, that it does not penetrate the cell. However, if it be assumed that permeability and deacylation are not in fact important factors, then these results suggest that there is no deficiency of activating-enzyme activity in ascites-tumour cells and perhaps in other types of tumours. This is supported by the observation that there was a marked increase in the production of acetoacetate and fatty acids; such a finding argues against an impairment in the activating-enzyme system, since acetyl-CoA formation is established to be the first step in the biosynthesis of these substances. This finding would also argue against any possible excessive destruction of ATP and DPN, which, of course, could retard the cycle activity in the tumour tissues employed.

In view of the present findings, together with those in the literature cited, it seems reasonable to conclude that the reactions of the Krebs cycle are of a lower order in the tumour as compared with normal tissues investigated. A diminished amount or activity, or both, of some intracycle enzyme system(s) appears to be the limiting factor.

SUMMARY

1. The operation of the Krebs cycle in certain tumour and non-tumour tissues has been studied by measuring the concentrations and ^{14}C-activities of the cycle intermediates when acetate, malate and/or succinate were used as substrates in the presence and absence of the blocking agents, fluoroacetate and malonate. The utilization of these substances by way of the citric acid cycle was less in the Walker, LC-18 and mouse ascites tumours than in muscle, liver (homogenates and isolated intact cells) or nucleated erythrocytes.

2. Addition of adenosine triphosphate and two concentrations of diphosphopyridine nucleotide to the incubation mixture, in the presence of fluoride or triethyltin acetate, did not result in any appreciable increase in the amounts or ^{14}C-activities of the citric acid-cycle members in tumours. Likewise, fortifying the incubation mixture with either coenzyme A or preformed acetyl-coenzyme A failed to produce a significant increase in the quantities and activities of the cycle acids. The only increase was in the lipid and acetoacetate fractions. This was considered to indicate that there was no apparent impairment in the activating-enzyme system or excessive loss of adenosine triphosphate or diphosphopyridine nucleotide in the tumours employed.

3. The cycle intermediates, succinate and malate, were not utilized any better by the tumour tissues than was acetate.

4. In view of the above findings, it is concluded that the lower activity of the cycle in the tumour than in the normal tissues studied is primarily due to some defect in an intra-Krebs-cycle reaction(s).

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The Metabolism of C_2-Compounds in Micro-organisms

8. A DICARBOXYLIC ACID CYCLE AS A ROUTE FOR THE OXIDATION OF GLYCOLLATE BY *ESCHERICHIA COLI**

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Although the tricarboxylic acid cycle has been established as the major route for the oxidation of acetate, and of compounds catabolized to acetate, in a wide variety of organisms (for review, see Krebs & Lowenstein, 1960) little is known of the pathways of oxidation of C_2 compounds more highly oxidized than acetate, such as glycollate or glyoxylate. The main purpose of this paper is to show that, whereas the tricarboxylic acid cycle plays an essential role in the growth of *Escherichia coli* on glycollate as sole carbon source, this cycle is not necessary for glycollate oxidation. The results obtained with the mutant M 22-64 of *E. coli*, strain *w*, which is devoid of the citrate-forming condensing enzyme (Gilvarg & Davis, 1957), and with its parent wild type, support the view that glyoxylate, derived from the oxidation of glycollate, can be oxidized completely via a dicarboxylic acid cycle in which glyoxylate condenses with acetyl-coenzyme A to form malate, which is oxidized via oxaloacetate and pyruvate to regenerate the acetyl-coenzyme A required for the initial condensation. An outline of this work has been published (Kornberg & Sadler, 1960).

MATERIALS AND METHODS

Maintenance and growth of micro-organisms. The micro-organisms used in this work were *E. coli*, strain *w*, and the mutant M 22-64 isolated and characterized by Gilvarg & Davis (1957). The mutant differed from its parent wild strain in being unable to catalyse the condensation of acetyl-coenzyme A and oxaloacetate to form citrate, and hence required the addition of glutamate for growth. The original cultures of the mutant and of the parent wild type were gifts from Professor B. D. Davis (Harvard Medical School, Boston, Mass., U.S.A.).

Cultures of the mutant and of the parent wild type were grown and maintained on agar slopes, containing 50 mM-potassium phosphate buffer, pH 7·2, 50 mM-NH_4Cl, 50 mM-sodium glycollate, 2 mM-sodium glutamate and essential salts (4 mg. of $CaCl_2,6H_2O$; 0·2 mg. of $MnCl_2,4H_2O$; 4 mg. of $MgSO_4,7H_2O$ and 0·2 mg. of $FeSO_4,6H_2O$ per 100 ml. of medium) solidified with 1·5% (w/v) of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in 400 ml. of the above medium with the agar omitted (hereafter referred to as 'glycollate–glutamate' medium) in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland). The flasks were shaken for 30–35 hr. on a reciprocal shaker at 30°. Growth of the cells was determined by measurements in an EEL Unigalvo Type 20 nephelometer (Evans Electroselenium Ltd., Harlow, Essex) of the light-scattering of samples of the bacterial suspensions and comparison of the observed readings with a previously constructed calibration curve relating such readings to the dry weight of bacteria/ml. of suspension. Although the medium was shown to be adequate to sustain the growth of more than 1 mg. dry wt. of bacteria/ml., the organisms were harvested while in the phase of logarithmic growth, at densities of 0·2–0·5 mg. dry wt./ml. The contents of the Carrel culture flasks were centrifuged at 10° for 15 min. at 1500*g*.

Oxidation studies. The packed cells were suspended in 100–150 ml. of potassium phosphate, pH 7·2, centrifuged at 1500*g* for 10 min., and resuspended in a sufficient volume of buffer to give a cell density of 5 mg. dry wt./ml. Samples (2 ml.) of this washed cell suspension were pipetted into the main compartment of Warburg manometer cups.

* Part 7: Gotto & Kornberg (1961).

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The centre wells contained 0·2 ml. of 2N-KOH and a strip of Whatman no. 40 paper. Substrates and inhibitors were placed in the main compartments or in the side arms as required (see Tables). In those experiments in which evolved labelled CO_2 was quantitatively collected for radioassay, double-armed cups were used: in these cases, the second side arm contained 0·3 ml. of aqueous 50% (w/v) trichloroacetic acid, and the paper strips were omitted from the centre wells.

All manometric experiments were performed at 30°, with air as the gas phase.

Bacterial incorporation of [^{14}C]*glycollate and* [^{14}C]*acetate.* Batches of the wild type or mutant cells which had been grown on the glycollate–glutamate medium were centrifuged and resuspended without washing in a sufficient quantity of buffer and salts similar to those supplied in the growth medium, but lacking the carbon substrates, to give a cell density of approximately 5 mg. dry wt./ml. Portions (20–30 ml.) of this suspension were placed, in a constant temperature bath at 30°, in a wide tube and aerated vigorously by forcing a stream of air through a sintered disk fused into its lower end. After 5 min., sufficient quantities of 1M-sodium glycollate and 1M-sodium acetate were added to give final concentrations of 20 and 10mM respectively (the acetate being added only in those experiments where the labelled substrate was to be acetate). After a further 5 min., labelled substrate was added and samples were taken at measured times.

The sampling procedure consisted in drawing 1·0 ml. portions of the oxidizing bacterial suspension into 5 ml. hypodermic syringes containing air and 0·2 ml. of [1-^{14}C]-glycollate (50 μc/ml.; 26 mM), or of [2-^{14}C]glycollate (50 μc/ml.; 26 mM), or of [2-^{14}C]acetate (100 μc/ml.; 17 mM). The syringes containing the samples were shaken manually and, after a measured time interval, their contents were ejected into tubes containing 3 ml. of absolute ethanol. For incubations of over 30 sec. duration, the needles of the syringes were plunged into corks, and the syringes were shaken while immersed in the constant-temperature bath at 30°.

Analysis of samples obtained from incubation experiments. The aqueous ethanolic suspensions were analysed by two-dimensional chromatography and radioautography. The methods used, and the procedures for identification of labelled compounds, and for radioassay, were as described by Kornberg (1958).

Assay of $^{14}CO_2$. Labelled CO_2 absorbed in the centre-wells of Warburg vessels was converted into $Ba^{14}CO_3$ by precipitation with $BaCl_2$, and its radioactivity was assayed, as described by Kornberg & Gotto (1961).

Adaptation studies with Escherichia coli, *strain* W. Suspensions of the wild-type organism, grown on either the glycollate–glutamate medium or the standard acetate medium used in previous work (Kornberg, Phizackerley & Sadler, 1960) were centrifuged at 1500*g* for 15 min. The packed cells were suspended to a final cell concentration of 0·2 mg. dry wt./ml. in two Carrel flasks immersed in a constant-temperature bath at 30°, one containing 200 ml. of the glycollate–glutamate medium and the other 200 ml. of the acetate medium. Growth of the cultures was followed nephelometrically as described above, and sufficient substrate was provided to allow for approximately three doublings in cell density. Samples (20 ml.) were removed at zero time and after every hour. These were centrifuged at 18000*g* for 3 min. at 3°, resuspended in 5·5 ml. of ice-cold 0·9% (w/v) KCl and immediately frozen at −15°. The samples taken were later thawed and used for the preparation of extracts.

Preparation of cell extracts. Extracts of cells were prepared by exposing suspensions of cells (1–20 mg. dry wt./ml.) in 0·9% KCl to the output of a 600 w Mullard magnetostrictor oscillator, operating at 3·5A, 25 kcyc./sec., for 3 min. The extracts were centrifuged at 18000*g* at 0° for 15 min. and the supernatant solutions were used. The protein content of such solutions was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of enzymes in cell-free extracts. Glycollic acid oxidase was measured as the rate of increase in extinction at 550 mμ concomitant with the reduction of cytochrome *c* in the presence of glycollate, enzyme and catalytic quantities of phenazine methosulphate (Gotto, 1961). The citrate-forming condensing enzyme (Ochoa, Stern & Schneider, 1951), malate synthetase (Wong & Ajl, 1956) and *iso*citratase (Smith & Gunsalus, 1954, 1957; Olson, 1954, 1959) were assayed by the spectrophotometric procedures of Dixon & Kornberg (1959). Malic dehydrogenase was estimated as described by Mehler, Kornberg, Grisolia & Ochoa (1948) and malic enzyme as described by Ochoa, Mehler & Kornberg (1948). Fumarase was measured by the method of Racker (1950). The pyruvic-oxidase system was assayed by the spectrophotometric procedure of Korkes (1955). The acyl thiol ester formed in this reaction was identified as acetyl-coenzyme A by its conversion into acetylhydroxamic acid, which was detected by chromatography (Stadtman & Barker, 1950). All spectrophotometric assays were carried out with a Cary model 14 recording spectrophotometer, at 23°.

Oxaloacetic-carboxylase activity (Utter & Kurahashi, 1954) was determined manometrically, by measuring the initial rate of CO_2 release from oxaloacetate under anaerobic conditions. Cups contained, in the main compartment, crude bacterial extract (containing 7·1 mg. of soluble protein), 100 μmoles of potassium phosphate buffer, pH 7·0, 10 μmoles of $CoSO_4$ and water to 2·6 ml. The side arm contained, in 0·5 ml., 40 μmoles of freshly dissolved, neutralized oxaloacetate, 10 μmoles of disodium adenosine triphosphate and 40 μmoles of potassium phosphate buffer, pH 7·0. After thermal equilibration at 30° for 10 min., the contents of the side arm were added to those of the main compartment. The manometers were read every 3 min. for 25 min.

Tests for reversion or contamination. Before use, all cultures of the mutant were tested to ensure the absence of the condensing enzyme. This was done either by spectrophotometric assay (Dixon & Kornberg, 1959) of sonic extracts, or by manometric tests for the ability of washed cell suspensions to oxidize acetate. Whereas the parent wild type readily oxidized acetate (Q_{O_2} = 60–70 μl. of O_2 absorbed/mg. dry wt./hr.), the mutant did not oxidize this substrate.

Materials. Purified sodium glyoxylate was a gift of Dr I. Zelitch (Connecticut Agricultural Experiment Station, New Haven, Conn., U.S.A.). Oxaloacetic acid and sodium pyruvate were gifts of Professor Sir Hans Krebs, F.R.S. Thiamine pyrophosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; adenosine triphosphate, pyridine nucleotides and coenzyme A from C. F. Boehringer und Soehne, Mannheim, Germany. Other

chemicals used were of the highest purity commercially available. Isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks.; they were purified before use as previously described (Kornberg, 1958; Kornberg & Gotto, 1961).

RESULTS

Growth on glycollate. The *E. coli* mutant M22-64, which required glutamate or α-oxoglutarate for growth, was isolated by Gilvarg & Davis (1957), who showed that its growth requirement was a consequence of the lack of the citrate-forming condensing enzyme.

The organism grew readily on a medium containing 50 mM-glycollate as main carbon source if this medium was supplemented with a small amount (2 mM) of glutamate, but did not grow in the absence of added glutamate. On this glycollate–glutamate medium, the mutant grew at approximately the same rate as did the parent wild type, *E. coli*, strain *w*: the mean generation times at 30° were, respectively, 229 ± 17 min. (eight experiments) and 190 ± 20 min. (four experiments). The growth yields of the two types of organisms were similarly not significantly different: under the conditions used, the medium supported an increase in dry weight of bacteria of 19·3 ± 0·3 μg./μmole of added glycollate (three experiments) for the mutant and 19·4 ± 0·3 μg./μmole of added glycollate (four experiments) for the wild type. These results, which confirm the findings of Gilvarg & Davis (1957) that *E. coli* cannot synthesize glutamate in the absence of the citrate-forming condensing enzyme, also indicate that, after relief of the enzyme deficiency of the mutant, both it and the wild-type organism utilized glycollate as a growth substrate by similar pathways.

Oxidation of glycollate and glyoxylate. Washed suspensions of the mutant, grown on the glycollate–glutamate medium, oxidized glycollate and glyoxylate at the same rate and to the same extent as did the parent wild type grown on this medium (Table 1). This indicates that the routes of oxidation of glycollate and glyoxylate are similar in the two types of organism, and shows that these routes are independent of a functioning tricarboxylic acid cycle.

Similar results were obtained with [^{14}C]glycollate. In both types of organism, the oxidation of [^{14}C]glycollate led to the evolution, as $^{14}CO_2$, of 93–98 % of the isotope in the carboxyl group, and 43–52 % of the isotope from the hydroxymethyl group.

2:4-Dinitrophenol (Clifton, 1946) acted in like manner on both mutant and wild-type organisms. Addition of this compound to 0·5–1 mM final concentration raised the quantity of oxygen absorbed by the wild type from 65 to 84 %, and by the mutant from 66 to 81 %, of that required for complete oxidation of glycollate, and also stimulated by 50 % the release of isotope as $^{14}CO_2$ from [2-^{14}C]glycollate.

The possibility that glycollate and glyoxylate oxidations proceeded via free formate was tested by the addition of 2·5 mM-hypophosphite, a specific inhibitor of formic dehydrogenase (Takamiya, 1953) to washed suspensions of mutant and wild-type organisms oxidizing glycollate, glyoxylate or formate. Formate oxidation, which was rapid and complete in the absence of this inhibitor, was abolished in its presence, but the oxidations of glycollate and glyoxylate were unaffected in rate or extent. This indicated that free formate was not an intermediate in their catabolism.

Incorporation of ^{14}C *from* [^{14}C]*glycollate.* When [1-^{14}C]- or [2-^{14}C]-glycollate was added to suspensions of the mutant or wild-type organisms oxidizing unlabelled glycollate, ^{14}C was rapidly incorporated into cellular materials soluble in aqueous ethanol. The rates of isotope incorporation were constant over the first 60–80 sec. of incubation, and corresponded to the utilization of [2-^{14}C]glycollate at the rate of 6 μmoles/hr./mg. dry wt. of mutant organism and of 7 μmoles/hr./mg.

Table 1. *Oxidation of glycollate and of glyoxylate by* Escherichia coli, *strain* w, *and its mutant M* 22-64

The main compartments of Warburg manometer flasks contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (5 mg. dry wt.). The centre wells contained 400 μmoles of KOH. Substrates as indicated were added from the side arms after thermal equilibration at 30°. Results are expressed as mean values ± standard deviation of the samples. The numbers of separate experiments are given in parentheses.

Quantity measured	Substrate	Organism used: *E. coli*, *w*	Organism used: M22–64
Extent of oxidation (% of theory for complete combustion)	Glycollate	61 ± 3 (3)	59 ± 7 (21)
	Glyoxylate	54 ± 7 (3)	57 ± 8 (8)
Q_{O_2} (μl. of O_2 absorbed/mg. dry wt./hr.)	Glycollate	111 ± 30 (3)	94 ± 23 (21)
	Glyoxylate	38 ± 7 (3)	45 ± 18 (6)
$Q_{substrate}$ (μmoles oxidized/mg. dry wt./hr.)	Glycollate	3·3 ± 0·9 (3)	2·8 ± 0·7 (21)
	Glyoxylate	1·7 ± 0·3 (3)	2·0 ± 0·8 (6)

dry wt. of wild-type cells. [1-^{14}C]Glycollate was incorporated at 3 μmoles/hr./mg. dry wt. by the mutant: this lower rate of incorporation would be expected from the rapid loss of isotope as $^{14}CO_2$ from the carboxyl group.

These rates are minimum ones, as neither volatile products such as carbon dioxide nor chromatographically fugitive materials such as oxaloacetate were assayed. They are, however, of the same order as those calculated from the observed rates of glycollate oxidation (Table 1) when these are corrected for assimilation.

Distribution of ^{14}C *amongst the labelled materials formed from* [^{14}C]*glycollate.* Two-dimensional chromatography and radioautography of the aqueous-ethanol fractions, obtained from each sample of the suspensions of mutant and wild-type organisms oxidizing glycollate, showed that isotope appeared rapidly in malate, fumarate, succinate, aspartate, alanine, carbohydrates and phosphorylated materials when the labelled substrate was either [1-^{14}C]- or [2-^{14}C]-glycollate. Labelled glycerate also appeared in all cases, but its contribution to the total amount of radioactivity varied and was greater for [2-^{14}C]- than for [1-^{14}C]-glycollate. Subsequent analysis of the area of phosphorylated material showed that, in the early samples at least, the major proportion of the isotope incorporated into this fraction was present in phosphoglyceric acid and phosphoenolpyruvate. No label was detected in citrate, α-oxoglutarate or glutamate in any sample from the mutant suspensions, though these compounds were heavily labelled in samples taken from the wild type. The general pattern of isotope distribution found was, with the exception of these compounds, identical in the experiments with either mutant or wild-type organisms: for simplicity, only the former are shown in Figs. 1 and 2.

When the proportions of the total radioactivity contributed by each compound, expressed as the percentage of the total isotope incorporated in each sample, were plotted against the times at which such samples were taken, smooth curves could be drawn through the points obtained (Figs. 1, 2). After approx. 150 sec., steady-state conditions had apparently been reached, as indicated by the

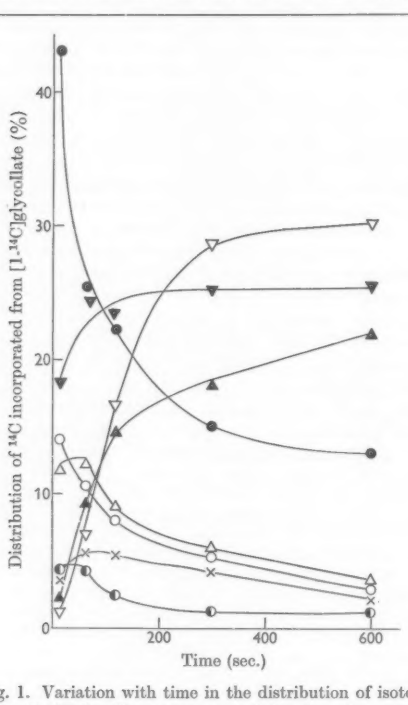


Fig. 1. Variation with time in the distribution of isotope from [1-^{14}C]glycollate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were polysaccharide (▽), phosphorylated materials (▼), glucose (▲), succinate (△), fumarate (○), alanine (×), aspartate (◐) and (predominating at early times) malate (●).

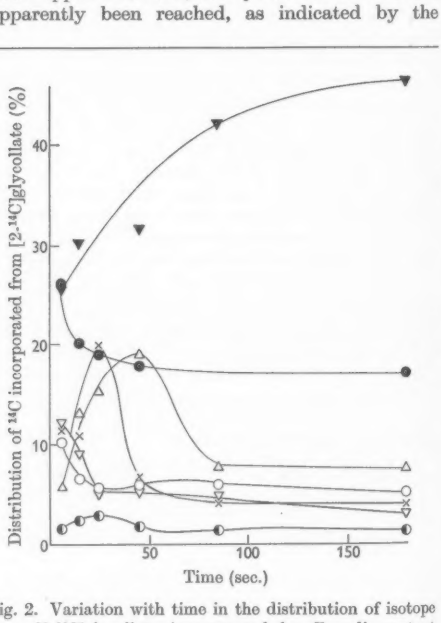


Fig. 2. Variation with time in the distribution of isotope from [2-^{14}C]glycollate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were phosphorylated compounds (▼), succinate (△), fumarate (○), alanine (×), glycerate (▽), aspartate (◐) and (predominating at the earliest times) malate (●).

relative constancy of isotope distribution amongst the components of the various samples. At and after this time, most of the incorporated isotope was found in carbohydrates and phosphorylated compounds, and 14–18% in malate. At earlier times, however, this distribution was reversed: malate contained the highest proportion of ^{14}C incorporated and, whereas the relative amounts of most other labelled materials initially rose with time, that of malate showed a continuous decrease. These results indicated that isotope from [^{14}C]glycollate was initially incorporated into malate by organisms oxidizing this labelled substrate; the initially low and rising proportions of isotope in succinate suggested that this compound had acquired label from malate and not vice versa. The negative slope of the glycerate curve indicated that isotope from glycollate also entered this material before passing on to other intermediates: this could be explained by the operation of a pathway for net synthesis of cell materials from glycollate similar to, or identical with, that found by Kornberg & Gotto (1959, 1961) with *Pseudomonas* spp.

Similar results were obtained with the wild-type organism oxidizing [1-^{14}C]- and [2-^{14}C]-glycollate, except that citrate and glutamate also became labelled. The relative proportion of the isotope incorporated into glutamate rose to a high level with time whilst that of citrate fell. Since neither of these materials was formed by the mutant, these results indicate that the tricarboxylic acid cycle functions in the wild-type organisms as a means of providing precursors of cell materials from glycollate rather than as a stage in glycollate oxidation.

Analysis of 2:4-dinitrophenylhydrazones prepared from all samples further showed that [^{14}C]-glycollate oxidation by the mutant resulted in rapid and heavy labelling of glyoxylate, oxaloacetate and pyruvate. At the earliest times, the bulk of the radioactivity was contained in glyoxylate, with oxaloacetate and pyruvate acquiring ^{14}C thereafter.

Effect of acetate on growth. Although the wild-type organism grew readily on acetate, the mutant did not grow on this carbon source even when the medium was supplemented with glutamate. This finding was expected as the growth of *E. coli* on acetate, which has been shown to be effected via the tricarboxylic acid and glyoxylate cycles (Kornberg, Phizackerley & Sadler, 1959, 1960; Reeves & Ajl, 1960), is not possible in the absence of a means of forming citrate. However, the addition of acetate (25 mM) to the glycollate–glutamate growth medium consistently stimulated the rate of growth of the mutant and reduced the mean generation time, by about 20%, to 183 min. (five experiments). Similarly, the addition to the glycollate–glutamate medium of acetate to a final concentration of 50 mM increased the growth yield of the mutant by 40%, to 29 μg. dry wt./μmole of added glycollate. Cells thus grown still failed to oxidize acetate alone and did not contain the citrate-forming condensing enzyme. This suggested that acetate, which, in the absence of glycollate, was not utilized by the organism, could enter metabolic pathways in its presence; it therefore indicated a point of entry into such routes other than via the condensing enzyme.

Incorporation of ^{14}C from [2-^{14}C]acetate. When [2-^{14}C]acetate was added to washed suspensions of either the mutant or wild-type organisms oxidizing unlabelled glycollate, ^{14}C was incorporated into cellular materials soluble in aqueous ethanol. The rates of isotope incorporation were constant over the first 90–100 sec. of incubation and corresponded to the uptake by respectively mutant and wild-type cells of 0·05 and 0·8 μmole of acetate/hr./mg. dry wt. The comparatively low rate of uptake of isotope from acetate is, in part, due to the small quantities of acetic thiokinase in glycollate-grown cells: the activation of [2-^{14}C]acetate to [2-^{14}C]-acetyl-coenzyme A would thus be expected to impose a rate-limiting step. The difference in the rates observed with the mutant and wild-type organisms indicates that, under these conditions, the major route for the utilization of exogenous acetate is via the tricarboxylic acid cycle.

Distribution of ^{14}C amongst the labelled materials formed from [2-^{14}C]acetate. With the exception of glutamate and citrate, which formed the bulk of labelled materials in the wild-type organisms but were absent from the mutant, the relative distribution of labelled products and their changes with time were similar with mutant and wild-type cells: again, for simplicity, only the results obtained with the mutant are shown in Fig. 3. As had been observed with [^{14}C]glycollate, one of the earliest labelled materials formed from [^{14}C]acetate was malate, which contained approx. 20% of the total isotope incorporated after 5 sec. incubation and the contribution of which continuously declined over the next 55 sec. to a steady-state level, at which it contained 8·4% of the total isotope incorporated. A similar high proportion of isotope was incorporated into aspartate, the contribution of which compound rose from an initial value of 19% at 5 sec. to a maximum of 21% at 23 sec., and declined continuously thereafter to a steady-state value at which it contributed 6% to the total radioactivity of the samples. Analysis of 2:4-dinitrophenylhydrazones prepared from each sample further showed that isotope from [^{14}C]-acetate rapidly appeared in oxaloacetate and pyruvate, but not in glyoxylate. It is therefore probable that, since oxaloacetate is fugitive on

two-dimensional chromatograms, the time course of labelling of aspartate is a reflexion of the rapid formation of labelled oxaloacetate from labelled malate.

These results show that the mutant, which, in the absence of glycollate cannot utilize acetate, can in its presence incorporate isotope from [^{14}C]-acetate into cellular materials, and that at the earliest times malate and oxaloacetate thus acquire label and in that order.

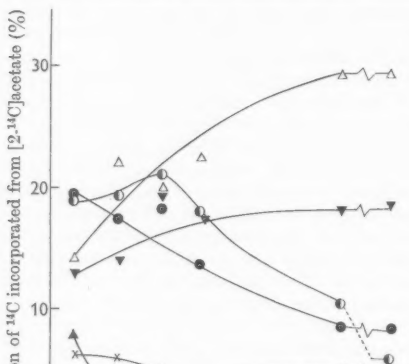


Fig. 3. Variation with time in the distribution of isotope from [2-^{14}C]acetate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were succinate (△), phosphorylated compounds (▼), aspartate (◐), alanine (×), glycine (▲), glycerate (▽) and (predominating at the earliest times) malate (●).

Effect of acetate on the oxidation of glycollate or glyoxylate. Although acetate, added as sole substrate, was not oxidized by washed suspensions of the mutant, the total quantities of oxygen absorbed during the oxidation of glyoxylate were raised consistently by 20 % when acetate was also added to various final proportions (Table 2). Moreover, the maximum rates of oxygen uptake observed showed a small but reproducible increase in the presence of acetate.

Effect of glycollate or glyoxylate on the oxidation of acetate. When washed suspensions of the mutant were incubated with [1-^{14}C]- or [2-^{14}C]-acetate, only traces of isotope (0·5 and 0·3 % respectively of that added) were evolved as labelled carbon dioxide. However, this small amount of acetate oxidation was stimulated 70–80-fold when unlabelled glyoxylate or glycollate was also added to suspensions of the mutant incubated with [1-^{14}C]-acetate: of the isotope added, 44 % was recovered as labelled carbon dioxide in the presence of glyoxylate, and 36 % in the presence of glycollate. Pyruvate was ineffective in promoting this oxidation of [1-^{14}C] acetate (Table 3).

Similar results were obtained with [2-^{14}C]-acetate (Table 4). Addition of unlabelled glycollate or glyoxylate to suspensions of the mutant incubated with [2-^{14}C]acetate raised the proportion of added isotope recovered as labelled carbon dioxide from 0·3 to 12 %. This stimulation was abolished by addition of arsenite, and was also not observed when pyruvate or L-malate was added instead of glycollate or glyoxylate.

Table 2. *Stimulation by acetate of the oxidation of glyoxylate by the mutant M* 22-64

The main compartments of Warburg manometer flasks contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (3–5 mg. dry wt.). The centre wells contained 400 μmoles of KOH. Substrates as indicated were added from the side arms after thermal equilibration at 30°.

Substrate	Quantity added (μmoles)	O_2 uptake observed (μl.)	Increase in O_2 uptake in the presence of acetate (μl.)	(%)
Acetate	20	0	—	—
Glyoxylate	10	116	—	—
Glyoxylate	10	139	23	20
Acetate	20			
Glyoxylate	5	69	—	—
Glyoxylate	5	83	14	20
Acetate	10			
Glyoxylate	5	82	13	19
Acetate	20			
Glyoxylate	4	62	—	—
Glyoxylate	4	75	13	21
Acetate	40			

Oxidation of L-*malate, oxaloacetate and pyruvate.* Washed suspensions of the wild-type organisms readily oxidized L-malate, oxaloacetate and pyruvate, the quantities of oxygen absorbed being respectively 70, 68 and 66 % of those required for complete oxidation. In contrast, washed suspensions of the mutant oxidized these substrates to a much smaller extent. Of the quantities of oxygen required for their complete oxidation, only 21 % was absorbed when L-malate or oxaloacetate were the substrates, and only 26 % when pyruvate was used. These quantities are respectively 62, 63 and 80 % of those required for the oxidation of these compounds to acetate.

Table 3. *Effect of added materials on* [1-^{14}C]*acetate oxidation by the mutant M* 22-64

The main compartments of double-armed Warburg manometer cups contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (10 mg. dry wt.). Sodium [1-^{14}C]acetate (6·8 μmoles; giving $1·3 \times 10^6$ counts/min. under the conditions of radioassay used) and unlabelled substrates (20 μmoles) as indicated were placed in one side arm, 0·3 ml. of 50 % (w/v) trichloroacetic acid in the second. The centre wells contained 400 μmoles of KOH. After thermal equilibration at 30° for 10 min., the contents of the first side arm were mixed with those of the main compartment. The incubation was continued until oxygen uptake ceased, after which time the contents of the second side arm were added. After a further 15 min., the cups were detached and the contents of the centre wells were precipitated as $BaCO_3$ for radioassay as described by Kornberg & Gotto (1961).

Unlabelled substrate added	$10^{-3} \times$ Radioactivity of $BaCO_3$ (counts/min.)	Fraction of added isotope recovered as $BaCO_3$ (%)
None	7	0·5
Pyruvate	4	0·3
Glycollate	466	36
Glyoxylate	575	44

Table 4. *Effect of added substrates on* [2-^{14}C]*acetate oxidation by the mutant M* 22-64

For conditions, see Table 3. Sodium arsenite was added to the contents of the main compartments to a final concentration of 10 mM where indicated. The sodium [2-^{14}C]-acetate added, 5·8 μmoles/cup, gave $7·8 \times 10^5$ counts/min. under the conditions of radioassay used.

Unlabelled materials added	$10^{-3} \times$ Radioactivity of $BaCO_3$ (counts/min.)	Fraction of added isotope recovered as $BaCO_3$ (%)
None	2·5	0·32
Arsenite	2·1	0·26
Glycollate	97	12·4
Glycollate + arsenite	2·7	0·34
Glyoxylate	91	11·7
Glyoxylate + arsenite	2·6	0·33
L-Malate	2·3	0·30
Pyruvate	2·4	0·31

Similar results were found with labelled malate. After incubation of [1-^{14}C]malate with washed suspensions of the mutant, 35 % of the added isotope was recovered as $^{14}CO_2$ and 10 % as oxaloacetate and pyruvate. Addition of unlabelled pyruvate or acetate did not significantly alter these quantities (Table 5). With [2:3-$^{14}C_2$]malate or [3-^{14}C]pyruvate, only 1·5 and 1·6 % respectively of the added isotope was evolved as $^{14}CO_2$.

However, as the results of the isotope-incorporation experiments had indicated labelled malate and oxaloacetate to be formed readily from labelled glycollate or labelled acetate, and as glycollate was oxidized (presumably via malate and oxaloacetate) to an extent much greater than suggested by the above results, it was probable that the presence of glycollate or glyoxylate promoted the oxidation of malate, oxaloacetate and pyruvate, as they promoted the oxidation of acetate. This is confirmed by the results shown in Table 5. Addition of unlabelled glycollate to suspensions of the mutant oxidizing [1-^{14}C]malate resulted in a 44 % increase in the quantities of $^{14}CO_2$ evolved, the total amounts of isotope released being raised from 35 to 50·5 % of those added. A like effect was noted after addition of unlabelled glyoxylate: this material stimulated the release of $^{14}CO_2$, derived from the oxidation of [1-^{14}C]malate, from 35 to 51 %; of [2:3-^{14}C]malate, from 1·5 to 8·5 %; and of [3-^{14}C]-pyruvate, from 1·6 to 10 % of the isotope added. With [1-^{14}C]malate, the addition of unlabelled glycollate or unlabelled glyoxylate also caused a decrease in the quantities of labelled oxaloacetate and pyruvate formed, which indicated that the unlabelled materials had been in part converted into these α-oxo acids in the course of these oxidations.

Malate synthetase and condensing enzyme in Escherichia coli. The finding that labelled malate was formed as the earliest labelled product by cells oxidizing either [^{14}C]glycollate or [^{14}C]acetate in the presence of unlabelled glycollate or glyoxylate, indicated that the malate was probably formed by the action of malate synthetase (Wong & Ajl, 1956), which catalyses, reaction (1):

$$\text{Acetyl-coenzyme A} + \text{glyoxylate} + H_2O \rightarrow \text{malate} + \text{coenzyme A} \quad (1)$$

Assay of cell-free extracts of the mutant and the wild-type organism, grown on a variety of substrates, confirmed that this enzyme was present in glycollate-grown cells in concentrations three to four times higher than in cells grown on acetate, on which substrate the enzyme is known to participate in the biosynthesis of cell materials (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957, 1958; Kornberg, Collins & Bigley, 1960; Kornberg, Phizackerley & Sadler, 1960). Moreover, glycollate-grown cells contained the citrate-forming con-

Table 5. *Effect of added substrates on oxidation of* [^{14}C]*malate and* [^{14}C]*pyruvate by the mutant M* 22-64

The conditions used were similar to those of Table 3, but each cup received 2–5 mg. dry wt. of washed cells. The labelled substrates were mixed with unlabelled carriers to a final quantity of 5 μmoles/cup.

Labelled substrate	10^{-3} × Radioactivity added (counts/min.)	Unlabelled compound added	Quantity of unlabelled compound added (μmoles)	10^{-3} × Radioactivity of $BaCO_3$ (%)	Fraction of added isotope recovered as $BaCO_3$ (%)	10^{-3} × Radioactivity of oxaloacetate and pyruvate	Fraction of added isotope recovered as oxaloacetate and pyruvate (%)
[1-^{14}C]Malate	22·8	None	0	7·98	35	2·22	9·7
[1-^{14}C]Malate	22·8	Acetate	10	7·80	34	2·28	10
[1-^{14}C]Malate	22·8	Pyruvate	10	8·07	35	1·98	8·7
[1-^{14}C]Malate	22·8	Glycollate	10	11·8	51	1·08	4·7
[1-^{14}C]Malate	22·8	Glyoxylate	10	10·8	47	1·14	5·0
[2:3-$^{14}C_2$]Malate	36·6	None	0	0·57	1·5	—	—
[2:3-$^{14}C_2$]Malate	36·6	Glyoxylate	20	3·04	8·5	—	—
[3-^{14}C]Pyruvate	130	None	0	2·13	1·6	—	—
[3-^{14}C]Pyruvate	130	Glyoxylate	20	13·0	10	—	—

densing enzyme in less than one-third the quantity observed in similar cells grown on acetate. This suggested that, when *E. coli* grows on glycollate, malate synthetase may function as an enzyme of a respiratory pathway in addition to its involvement in a biosynthetic route.

This is supported by studies of the changes of the intracellular concentration of malate synthetase and the citrate-forming condensing enzyme during the adaptation of wild-type organisms to growth on acetate or glycollate (Fig. 4). Continued growth of acetate-grown cells on acetate was accompanied by a maintenance of the ratio of activities of malate synthetase to condensing enzyme at 0·43 throughout an increase of cell density from 0·2 to 1·6 mg. dry wt./ml. (Fig. 4, curve *C*). However, when acetate-grown cells continued to grow on glycollate (Fig. 4, curve *A*) this ratio changed from an initial value of 0·3 to a final value of 2·1. Similarly, when glycollate-grown cells continued to grow on acetate (Fig. 4, curve *B*) the ratio of activities of malate synthetase to condensing enzyme fell from an initial value of 1·7 to a final figure of 0·5.

Enzymes oxidizing malate in glycollate-grown Escherichia coli. The rapid labelling of oxaloacetate,

Fig. 4. Effect of change of growth substrate on the ratio of the activities of malate synthetase to condensing enzyme in extracts of *E. coli*, strain *w*. For details, see Materials and Methods. The ratio increased when acetate-grown cells continued to grow on glycollate (●, *A*), decreased when glycollate-grown cells continued to grow on acetate (○, *B*) and remained constant when acetate-grown cells continued to grow on acetate (×, *C*).

Table 6. *Enzymic activities in extracts of* Escherichia coli *strain* w *and M* 22-64 *grown on the glycollate–glutamate medium*

The extracts were prepared and the enzymes were assayed as described under Materials and Methods. Specific activities are expressed as μmoles of substrate transformed/mg. of soluble protein/hr.

Enzyme	Specific activity in *E. coli*, *w*	Specific activity in M 22–64
Glycollic acid oxidase	6	6
Malate synthetase	25	21
Malate dehydrogenase	70	70
Oxaloacetic carboxylase	15	13
Pyruvate oxidase	10*	6*
Fumarase	52	32

* Minimum values, as the DPNH-oxidase activity of *E. coli* extracts was too high for precise quantitative assay.

phosphopyruvate and pyruvate noted during the oxidation of labelled glycollate, or labelled malate, or labelled acetate in the presence of unlabelled glycollate or glyoxylate, suggested that the malate formed from acetyl-coenzyme A and glyoxylate, by reaction (1), was oxidized via oxaloacetate, phosphopyruvate and pyruvate to re-form acetyl-coenzyme A. Enzymes capable of catalysing these oxidations were present in high activity in extracts of both mutant and wild-type cells grown on the glycollate–glutamate medium (Table 6). The enzymic activities observed were 3–25 times greater than required to account for the observed rates of glycollate or glyoxylate oxidation by intact cells.

DISCUSSION

Little is known of the metabolic routes whereby micro-organisms oxidize glycollate or glyoxylate to carbon dioxide and water. A possible pathway might involve a stepwise degradation of glycollate via glyoxylate and formate (reactions 2–4).

$$\text{Glycollate} + \tfrac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O \quad (2)$$

$$\text{Glyoxylate} + \tfrac{1}{2}O_2 \rightarrow \text{formate} + CO_2 \quad (3)$$

$$\text{Formate} + \tfrac{1}{2}O_2 \rightarrow CO_2 + H_2O \quad (4)$$

The occurrence in glycollate-grown micro-organisms of the first enzyme of this sequence, glycollic acid oxidase (reaction 2), has been established (Kornberg & Gotto, 1959, 1961; Table 6), and the enzyme has been shown to be formed only in organisms grown on glycollate (Gotto, 1961). Evidence on the mechanism of the further oxidation of glyoxylate is sparse and confusing. Although the glycollic acid-oxidase system of plants seemed also to catalyse the oxidation of glyoxylate to formate and carbon dioxide (Claggett, Tolbert & Burris, 1949), this oxidation was shown to be a non-enzymic decomposition of glyoxylate by hydrogen peroxide produced in the oxidation of glycollate (Kenten & Mann, 1952; Kolesnikov, 1952; Zelitch & Ochoa, 1953). The formation of [^{14}C]formate and unlabelled carbon dioxide from [1-^{14}C]glyoxylate was observed by Campbell (1954, 1955) when extracts of a strain of *Pseudomonas* were shaken in air for 6 hr. in the presence of sodium hypophosphite, which was added to inhibit the oxidation of formate. It is difficult to envisage the mechanism of this reaction, which was stimulated by Mg^{2+} ions and thiamine pyrophosphate and which effected the transformation of the carboxyl group of glyoxylate to formic acid with concomitant oxidation of the aldehyde group to carbon dioxide: it is conceivable that this is not a one-step reaction but that it might involve a process analogous to the conversion of $^{13}CH_2(OH)\cdot{}^{14}CH_2(NH_2)$ into $^{13}CH_2(NH_2)\cdot{}^{14}CO_2H$ observed by Weissbach & Sprinson (1953) in rats. The oxidation of glyoxylate to formate and carbon dioxide has also been reported as an intermediate step in the oxidation of glyceric acid by a soil diphtheroid (Taylor & Juni, 1959).

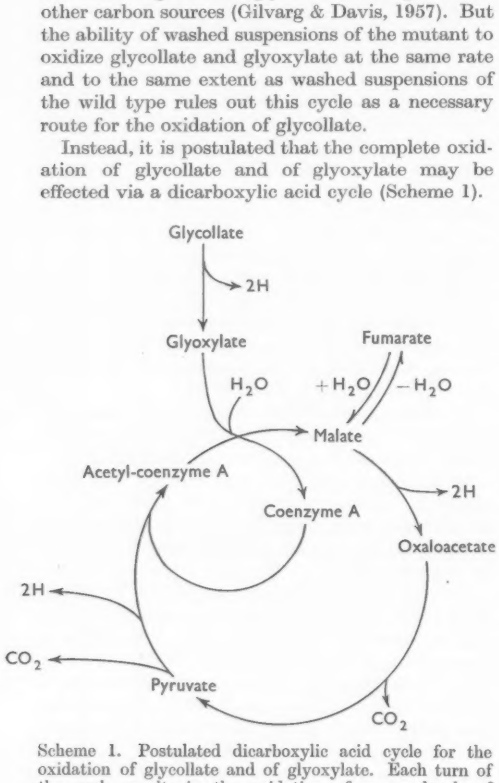
In contrast with these observations, Bachrach (1957) and Kornberg & Gotto (1959, 1961) failed to detect formate as a product of glyoxylate oxidation by several strains of *Pseudomonas*. Similarly, although sodium hypophosphite abolished the oxidation of formate by *E. coli*, *w* or M 22-64, this compound did not affect the oxidation of glycollate or of glyoxylate.

The inhibition of glycollate oxidation by fluoroacetate, observed by Kalnitsky & Barron (1947), and the accumulation of citrate as a consequence of this inhibition (Jayasuriya, 1955, 1956), are strong indications that the tricarboxylic acid cycle is involved in the microbial metabolism of glycollate. However, it is unlikely that this cycle functions as the main respiratory pathway under these conditions. Jayasuriya (1955, 1956), in an extensive investigation of the effect of fluoroacetate on the metabolism of *Pseudomonas* OD1, noted that, although isotope from [2-^{14}C]glycollate and (to a lesser extent) from [1-^{14}C]glycollate was incorporated into the citrate accumulated as a consequence of fluoroacetate inhibition, the carbon dioxide evolved from the oxidation of the labelled glycollate contained isotope at a specific activity much higher than that contained in the carboxyl groups of citrate, which are the sources of the carbon dioxide evolved in the tricarboxylic acid cycle.

Similar conclusions may be drawn from the experiments reported in this paper. The rapid incorporation of isotope from [^{14}C]glycollate or from [^{14}C]acetate into citrate and glutamate by *E. coli*, strain *w*, simultaneously oxidizing unlabelled glycollate indicates that the tricarboxylic acid cycle operates under these conditions. The failure

of the mutant M22-64, which lacks the citrate-forming condensing enzyme, to grow on glycollate unless the medium is supplemented with glutamate further shows that the tricarboxylic acid cycle is as essential for growth on glycollate as for growth on other carbon sources (Gilvarg & Davis, 1957). But the ability of washed suspensions of the mutant to oxidize glycollate and glyoxylate at the same rate and to the same extent as washed suspensions of the wild type rules out this cycle as a necessary route for the oxidation of glycollate.

Instead, it is postulated that the complete oxidation of glycollate and of glyoxylate may be effected via a dicarboxylic acid cycle (Scheme 1).



Scheme 1. Postulated dicarboxylic acid cycle for the oxidation of glycollate and of glyoxylate. Each turn of the cycle results in the oxidation of one molecule of glyoxylate:

$$CHO \cdot CO_2H + O_2 \longrightarrow 2CO_2 + H_2O$$

The evidence for this cycle rests on five main considerations:

(1) Isotope from labelled glycollate is incorporated into glyoxylate and into the intermediates of the cycle in the expected sequence.

(2) Although acetate alone cannot be utilized by the mutant, acetate stimulates both growth on, and the oxidation of, glycollate.

(3) In the presence of unlabelled glycollate, labelled acetate is incorporated into the intermediates of the cycle but not into glyoxylate.

(4) Extracts of glycollate-grown *E. coli* contain the enzymes of the cycle in activity greater than required to account for the rates of oxidation of glycollate by whole cells.

(5) Labelled acetate alone is not oxidized by the mutant, but is oxidized in the presence of glycollate or glyoxylate.

Moreover, the relative activities of malate synthetase and condensing enzyme in glycollate-grown *E. coli*, strain *w*, and their variations with change of growth substrate (Fig. 4), suggest that the dicarboxylic acid cycle effects the oxidation of glycollate whereas the tricarboxylic acid cycle supplies the carbon skeletons of cell materials synthesized during growth on glycollate. Many of the enzymic steps of the dicarboxylic acid cycle are also those of the tricarboxylic acid cycle, but an inverse relationship emerges between oxidations effected by these two cycles. Whereas in each turn of the tricarboxylic acid cycle one molecule of acetate (entering the cycle as acetyl-coenzyme A) is totally oxidized with the obligatory participation of an α-oxo acid (which is regenerated), in each turn of the dicarboxylic acid cycle, one molecule of α-oxo acid is totally oxidized with the obligatory participation of acetyl-coenzyme A (which is regenerated).

Although this dicarboxylic acid cycle has been postulated to participate in the oxidation of glycollate and of glyoxylate by several strains of *Pseudomonas* (Hullin & Hassall, 1960; Kornberg & Gotto, 1961) and has been shown to be a quantitatively important route for glycollate oxidation by *E. coli*, strain *w*, and its mutant M22-64, it is likely that it is not the only route for the microbial oxidation of these C_2 compounds. For example, an organism isolated from soil has been found to grow readily on glycollate, and to be rich in the enzymes which effect the biosynthesis of cell materials from glycollate (Kornberg & Gotto, 1961), but to contain relatively small amounts of malate synthetase (C. T. Gray & H. L. Kornberg, unpublished experiments). The nature of such alternative routes is under investigation.

SUMMARY

1. *Escherichia coli*, strain *w*, grew readily in a medium containing 50 mM-glycollate as carbon source. The mutant M22-64 (Gilvarg & Davis, 1957), devoid of the citrate-forming condensing enzyme, failed to grow in this medium unless small quantities (2 mM) of glutamate were also added.

2. The mutant and the wild-type organisms, grown on 50 mM-glycollate plus 2 mM-glutamate, oxidized glycollate or glyoxylate at the same rate and to the same extent. These oxidations were not affected by 2·5 mM-hypophosphite, which abolished formate oxidation.

3. Isotope from [1-^{14}C]- or [2-^{14}C]-glycollate was incorporated at early times, by organisms oxidizing unlabelled glycollate, into glyoxylate, malate,

oxaloacetate and pyruvate. Under these conditions, isotope from [2-^{14}C]acetate appeared in malate, oxaloacetate and pyruvate.

4. Although the mutant did not oxidize acetate, acetate stimulated the rate and extent of oxygen uptake by cells oxidizing glyoxylate.

5. Washed suspensions of the mutant evolved, as $^{14}CO_2$, 0·5% of the isotope from [1-^{14}C]acetate. This was increased 70–80-fold when unlabelled glycollate or glyoxylate was also added. Pyruvate was ineffective in promoting this oxidation of [1-^{14}C]acetate. Similar results were obtained with [2-^{14}C]acetate.

6. Addition of unlabelled glycollate or glyoxylate stimulated the release of isotope as $^{14}CO_2$ from [1-^{14}C]malate, [2:3-$^{14}C_2$]malate and [3-^{14}C]pyruvate.

7. These results suggest the operation of a dicarboxylic acid cycle, in which glyoxylate is totally oxidized with the catalytic participation of malate, oxaloacetate, pyruvate and acetyl-coenzyme A.

8. Measurement of the absolute levels of enzymes in glycollate-grown cells, and alterations in their relative proportions when cells adapt to grow on glycollate, support the occurrence of this postulated cycle.

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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

4. THE LOCALIZATION OF METABOLIC CHANGES WITHIN SUBCELLULAR FRACTIONS OF KREBS II MOUSE-ASCITES-TUMOUR CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS*

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Martin, Malec, Sved & Work (1961*b*) showed that, during a single growth cycle, infection with encephalomyocarditis virus caused no changes in the total deoxyribonucleic acid, ribonucleic acid or protein of the host ascites-tumour cell. However, by the use of [6-^{14}C]orotic acid and [^{14}C]valine, it was shown that there were substantial changes in the rates of turnover of ribonucleic acid and protein at different times during the cycle of virus growth. In particular, about 5 hr. after infection there was a striking increase in the rate of turnover of ribonucleic acid, and this increase coincided in time with the appearance of new virus. However, this stimulation in turnover appeared to be quantitatively greater than could be accounted for by formation of new virus ribonucleic acid.

Methods have now been developed for the well-defined separation of disrupted Krebs II ascites-tumour cells into nuclei, mitochondria, microsomes and cell sap (Martin, Malec, Coote & Work, 1961*a*). By the use of these methods we have been able to investigate the effect of virus infection on the turnover of the ribonucleic acid and protein of the subcellular components of the host cell, and thus to localize more exactly the sites of metabolic change within the infected cell. This paper describes the result of such a study.

METHODS

The origin and propagation of both the Krebs II mouse-ascites-tumour cells and the encephalomyocarditis virus have been described by Martin *et al.* (1961*b*).

Conditions of infection. Sufficient virus was added to suspensions of washed ascites-tumour cells in Earle's medium (2–3 $\times 10^7$ cells/ml.) to infect all cells (approx. 3 plaque-forming units/cell), and the cells were dispensed in 10 ml. portions into incubation flasks. The virus was allowed to adsorb for 30 min. at room temperature, followed by 15 min. at 36°. Suspensions of control (uninfected) cells were treated similarly. The cells were then incubated at 36° as described by Martin *et al.* (1961*b*).

For the study of protein and nucleic acid turnover, ^{14}C-labelled precursors were added at appropriate intervals during the virus growth cycle to flasks containing infected and control cells; 30 min. later the flasks were removed and diluted with an ice-cold solution of the unlabelled precursor in phosphate-buffered saline (Martin *et al.* 1961*b*), and the cells sedimented by centrifuging at 120*g* for 5 min. They were then washed twice with buffered saline and stored in an ice bath until required for disruption.

Disruption of Krebs II cells and fractionation of subcellular components. Cells were prepared for disruption by washing with calcium- and magnesium-free buffered saline (Martin *et al.* 1961*b*), then with 0·125 M-sucrose–0·075 M-KCl solution. The cells were disrupted by a combination of double osmotic shock and Potter homogenizer, as described by Martin *et al.* (1961*a*). Nuclei, mitochondria, microsomes and cell sap were separated from the tumour-cell homogenates by differential centrifuging in 0·25 M-sucrose–0·1 M-KCl solution (Martin *et al.* 1961*a*). Preparations of isolated nuclei were usually examined microscopically after staining with nigrosin, and were always found to contain less than 3% of whole-cell contamination. The intermediate fraction that separated between nuclei and mitochondria, and which represented only a very small percentage of the cell contents, was discarded. Mitochondria were washed once with sucrose–KCl solution, but the microsomal fraction was not further treated after isolation except to rinse the surface of the pellet with distilled water.

Estimation of virus. Virus was estimated in tumour-cell homogenates and in the subcellular fractions derived from them by both haemagglutinin titration and plaque assay, as described by Martin *et al.* (1961*b*). To release any adsorbed virus, the nuclear fractions were incubated at 32° for 1 hr. with deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; 0·1 mg./ml.) before virus assay.

Estimation of protein and nucleic acids. Protein, RNA and DNA were isolated from tumour-cell homogenates, mitochondria, microsomes and cell sap and estimated as described by Martin *et al.* (1961*b*). The extraction methods were not considered ideal for the isolation and estimation of RNA from the nuclear fraction. Therefore the nuclei, free of lipids and material soluble in cold 0·2 N-$HClO_4$, were treated with 0·3 N-NaOH at 37° for 18 hr. to hydrolyse RNA, and protein and DNA were precipitated by addition of $HClO_4$. The DNA was removed from the acid-insoluble precipitate by extraction with 0·5 N-$HClO_4$ at 70° for 30 min. or, when required for the estimation of specific

* Part 3: Martin, Malec, Coote & Work (1961*a*).

Table 1. *Specific radioactivities of Krebs cell protein and nucleic acids after incubation with* [^{14}C]*valine and* [6-^{14}C]*orotic acid and a mixture of both labelled compounds*

Flasks containing 10^8 ascites-tumour cells in 5 ml. of Earle's medium and the indicated ^{14}C-labelled compounds were incubated for 30 min. at 36° (Martin, Malec, Sved & Work, 1961*b*). Total nucleic acid and protein extracts were prepared and their radioactivities determined, as described in the text.

^{14}C-compound added	Time of incubation (min.)	Specific radioactivities: Protein (μmc/g.)	Specific radioactivities: Nucleic acids (μc/mole of nucleic acid P)	Ratio of specific activities: Nucleic acid / Protein
[^{14}C]Valine (0·1 μc)	30	276	27	2·59
[6-^{14}C]Orotic acid (5 μc)	30	3	714	
[^{14}C]Valine plus [6-^{14}C]orotic acid	30	266	665	2·50
[^{14}C]Valine plus [6-^{14}C]orotic acid	0	3	2	—

radioactivity, by extraction with 10% (w/v) NaCl solution (buffered at pH 6·0 with 0·1 M-sodium acetate). DNA was recovered from the hot saline extract by precipitation with 2·5 vol. of ethanol.

Measurement of radioactivity. Since the amount of work involved in cell disruption, in fractionation of the subcellular particles and in the separation of RNA and protein is rather considerable, it was thought best to study the incorporation of radioactive precursors (in this case [6-^{14}C]orotic acid and [^{14}C]valine) into both RNA and protein simultaneously. Before this could be done, however, it was necessary to show that cross-contamination of nucleic acid with protein, or vice versa, would not cause any significant error in the estimation of specific radioactivity. Ascites-tumour cells were therefore incubated in media containing either [6-^{14}C]orotic acid or [^{14}C]valine or a mixture of both labelled compounds. After incubation under the standard conditions, the appropriate ^{12}C carrier was added, and the protein and nucleic acid were assayed for radioactivity by the following method. The cells were resuspended in 0·1 M-DL-valine solution, $HClO_4$ was added to a concentration of 0·2 M, and the acid-insoluble material treated with an aqueous solution of trimethylamine (0·5 M) containing DL-valine (0·07 M) for 20 min. at 55°. The material did not completely dissolve, but the suspension became translucent. The suspension was cooled to 0°, $HClO_4$ added to a concentration of 0·2 M, and the precipitated material centrifuged off. The supernatant was examined for the presence of extracted nucleotides. Only about 3% of the total RNA was extracted during the trimethylamine treatment. The precipitate was washed twice with ice-cold 0·2 M-$HClO_4$. Total nucleic acids and protein were then extracted, and their specific radioactivities determined as described by Martin *et al.* (1961*b*).

From the results of this experiment (Table 1) it was concluded that there was no contamination of protein by the labelled orotic acid, but that slight contamination (about 4%) of the nucleic acid with labelled valine may occur. In a further experiment, Krebs cells were incubated for 30 min. with a fixed amount of [6-^{14}C]orotic acid (5 μc/flask containing 10^8 cells in 5 ml. of medium) and various amounts of [^{14}C]valine (0·02–0·2 μc/flask). When the radioactivity of the nucleic acid and protein was measured it was found that the slight contamination of the nucleic acid extract with valine was directly proportional to the specific activity of the protein fraction. It was thus possible to apply a suitable correction factor to the measured specific activity of nucleic acid to account for any contaminating radioactive amino acid present in the extract. In experiments where both labelled precursors were used simultaneously, two control incubations were therefore included:

(1) A flask in which the cells were incubated for the full period with [^{14}C]valine, then [6-^{14}C]orotic acid added just before the carrier. The radioactivity of the nucleic acids from these cells gave a measure of valine contamination, and the extent of this contamination was assumed to be proportional to the specific radioactivity of the protein fraction.

(2) A flask in which cells were incubated with [6-^{14}C]-orotic acid for the full period, then [^{14}C]valine added just before the carrier. The specific radioactivity of the protein from these cells was used as the zero-time control for estimates of protein radioactivity.

EXPERIMENTAL AND RESULTS

Effect of infection on incorporation of precursors into the protein and nucleic acids of subcellular components from tumour cells. A series of infected and control cultures of Krebs II ascites-tumour cells were incubated in Earle's medium under identical conditions. Exactly 30 min. before removing each flask from the incubation chamber, a mixture of [^{14}C]valine and [6-^{14}C]orotic acid was added. Flasks were removed at hourly intervals throughout the major portion of the virus growth cycle, ^{12}C carrier was added, and the cells were cooled at 0°, washed and disrupted by the double-osmotic-shock method (Martin *et al.* 1961*a*). Portions of the whole lysate were saved and the remainder was subjected to differential centrifuging in 0·25 M-sucrose–0·1 M-KCl. Protein and RNA were isolated from the nuclear, mitochondrial, microsomal and cell-sap fractions, as described under Methods, and their specific radioactivities were determined. Specific radioactivity measurements were also made on DNA from the nuclei. The results are summarized in Table 2. Difficulty was experienced in obtaining a representative sample of the nuclear protein by the method used (cf. Martin *et al.* 1961*a*), and no figures for the turnover rate of protein in the nuclear fraction are

given. As each subcellular fraction was isolated from the infected cells, part was set aside and its virus content assayed by the haemagglutination technique. The results of these assays are also given in Table 2.

Haemagglutinin is to a great extent concentrated in the mitochondrial fraction and appears in the microsomal fraction only in smaller amounts (Tables 2 and 4). At no time during the infection cycle were significant amounts of haemagglutinin found in the cell-sap fraction, and the trace of haemagglutinin found in the nuclear fraction was in part due to interference with the haemagglutinin reaction at low dilutions by DNA from the nucleus and in part to slight contamination with mitochondria.

The percentage of virus in the mitochondrial fraction of homogenates prepared by osmotic shock varied in successive experiments (67% of total in the experiment reported in Table 2, and 95% in the experiment of Table 4), but this probably reflects the impossibility of exact duplication of any method of cell rupture rather than biological variation.

The association of virus with the mitochondria was unexpected. It is certainly not caused by the sedimentation characteristics of the viral particle, since virus added to the cell homogenate before fractionation appears almost exclusively in the microsomal fraction after separation by differential centrifuging. As a further check, we have disrupted Krebs cells by an alternative method

Table 2. *Effect of encephalomyocarditis-virus infection on the incorporation of* [^{14}C]*valine and* [6-^{14}C]*orotic acid into the proteins and nucleic acids of ascites-tumour subcellular fractions*

Portions (10 ml.) of control and infected (3 plaque-forming units of virus/cell) suspensions of ascites-tumour cells (3×10^7 cells/ml.) were incubated at 36° for the various times indicated under conditions described by Martin *et al.* (1961*b*). No attempt was made at synchronizing the infectious process. [^{14}C]Valine (0·5 μc) and [6-^{14}C]orotic acid (25 μc) were then added as a solution in 1·0 ml. of buffered saline, and the flasks incubated for a further 30 min. The cells were washed, disrupted by osmotic shock and separated into the various subcellular fractions. Portions of each fraction were diluted with distilled water, stored overnight at 4° and assayed for haemagglutinating activity. Protein, RNA and DNA were isolated from the remaining portions and their specific radioactivities determined. All estimates of RNA specific activity have been corrected for contamination with valine, and of protein specific activity for contamination with orotic acid. The virus haemagglutinin titres are given in total haemagglutinin units/flask (3×10^8 cells), see Martin *et al.* (1961*b*).

Flask		Time after inoculation with virus (hr.) 1	2	3	4	5	6
Nuclear fraction							
RNA (μc/m-mole of RNA P)	Control	55·8	31·4	32·1	30·1	30·3	31·7
	Infected	45·3	15·9	12·2	5·6	7·8	3·2
DNA (μc/m-mole of DNA P)	Control	0·100	0·088	0·073	0·074	0·056	0·054
	Infected	0·108	0·083	0·058	0·043	0·042	0·019
Virus haemagglutinin titre	Infected	200	200	200	200	250	2050
Mitochondrial fraction							
Protein (μc/g.)	Control	1·33	1·08	0·93	0·82	0·72	0·66
	Infected	1·48	1·01	0·91	0·57	0·46	0·65
RNA (μc/m-mole of RNA P)	Control	1·22	0·910	0·775	0·595	0·565	0·610
	Infected	1·04	0·780	0·620	0·665	0·655	1·98
Virus haemagglutinin titre	Infected	0	0	0	1100	1100	36000
Microsomal fraction							
Protein (μc/g.)	Control	1·16	1·04	1·03	0·99	0·99	0·76
	Infected	1·24	1·08	0·84	0·73	0·52	0·47
RNA (μc/m-mole of RNA P)	Control	0·487	0·315	0·308	0·310	0·324	0·323
	Infected	0·448	0·206	0·140	0·165	0·189	0·545
Virus haemagglutinin titre	Infected	0	0	0	35	70	18000
Cell-sap fraction							
Protein (μc/g.)	Control	0·84	0·68	0·62	0·49	0·53	0·43
	Infected	0·93	0·79	0·66	0·43	0·30	0·24
RNA (μc/m-mole of RNA P)	Control	3·19	2·55	1·74	1·62	1·53	1·92
	Infected	2·68	1·98	1·07	1·22	1·38	2·05
Virus haemagglutinin titre	Infected	0	0	0	0	0	40

(Dounce homogenizer in 10 mM-$MgCl_2$; see Martin *et al.* 1961*a*), and again found most of the haemagglutinin in the mitochondrial fraction. Bellett & Burness (1960) have also reported the concentration of haemagglutinin in the mitochondrial fraction of Krebs II cells 4·5–6 hr. after infection with encephalomyocarditis virus.

When infected cells were disrupted by ultrasonic vibrations as described by Martin *et al.* (1961*a*), the virus was found largely in the microsomal fraction. In keeping with this observation it was found that virus could be released from mitochondria by treatment with ultrasonic vibrations.

When these experiments were designed it was assumed that haemagglutinin could be equated with virus. This assumption is supported by our demonstration (Faulkner, Martin, Sved, Valentine & Work, 1961) that the ratio of haemagglutinin to infectivity is the same in crystalline encephalomyocarditis virus as it is in crude virus preparation. This view is further strengthened by the results shown in Table 4, which indicate that haemagglutinating activity is associated with infective virus both in time of appearance during the growth cycle and in the site within the cell of maximum virus concentration. More precise measurements (E. M. Martin & T. S. Work, unpublished work) show that a slight delay occurs between the formation of viral protein and the appearance of an equivalent haemagglutinating activity, and this again suggests that haemagglutinating activity is a measure of the whole infective virus particle rather than of partially completed forms.

There are large differences in the rates of incorporation of orotic acid and valine into the different subcellular fractions of normal (control) Krebs II cells (Table 2). The rates of valine incorporation into the proteins of nuclei, mitochondria, microsomes and cell sap are in the proportions 0·28:1·0:1·2:0·67, whereas the rates of orotic acid incorporation into the ribonucleic acids of the same fractions are in the proportions 50:1·0:0·5:3·0. By using the data of Martin *et al.* (1961*a*) for the distribution of protein and RNA among these fractions, it can be calculated that turnover of RNA in the nucleus accounts for 88 % of the total cell RNA turnover, whereas mitochondria, microsomes and cell sap contribute 24, 33 and 39 % respectively to the total turnover of protein.

Infection with encephalomyocarditis virus caused an initial slight stimulation of valine incorporation into the proteins of all fractions. This stimulation was observed with whole-cell preparations (Martin *et al.* 1961*b*). The effect was most marked in the cell sap, thus supporting the suggestion that this initial stimulation in protein synthesis may represent the synthesis of new enzymes necessary for viral replication.

There then follows a period of general inhibition of protein turnover. The inhibition continued progressively during the course of the experiment in all fractions except the mitochondria, which showed marked increase in turnover rate from 5 hr. after infection. It is reasonable to suppose that this stimulation of protein turnover is associated with the appearance of virus in this fraction, and it is possible that it represents the incorporation of valine into the viral protein, as 6–6·5 hr. after infection is the period of maximal viral-protein synthesis under the growth conditions used in the present series of experiments.

Quantitative changes in ribonucleic acid in nuclei and mitochondria during virus infection

Martin *et al.* (1961*b*) showed that there was no significant change in the overall composition of infected Krebs cells as compared with normal controls. The marked fall in RNA turnover within nuclei of infected cells and the threefold increase in rate of turnover of RNA in the mitochondria of infected cells towards the end of the infectious cycle prompted the thought that there might have been quite substantial changes in the overall composition of subcellular fractions, but that, by chance, these had balanced one another and so produced the apparent overall constancy of composition observed earlier (Martin *et al.* 1961*b*). This view was strengthened by the results of the experiment described in Table 2, when it was observed that the net recovery of RNA from the mitochondrial fraction of cells in the later stages of infection was far higher than their corresponding controls, whereas the RNA to DNA ratio in infected nuclei appeared to fall. Accordingly, an experiment was set up to settle this point.

Krebs cells were harvested, washed and suspended in Earle's medium in the usual way (Martin *et al.* 1961*b*). The cell suspension was infected with encephalomyocarditis virus. All flasks were left at room temperature for 30 min. and then incubated at 36° under the standard conditions; one control and one infected flask were removed at 2 hr., another pair at 4 hr. and the last pair at 6·5 hr. The cells were collected, washed and disrupted by the double-osmotic-shock method (Martin *et al.* 1961*a*). The nuclear and mitochondrial fractions were isolated in the usual way, but the microsome fraction was not separated from the cell sap. The nuclear fractions were analysed for RNA and for DNA (see Methods), and the mitochondrial fractions were analysed for protein and for RNA (Table 3). In addition, the cytoplasmic fractions were assayed both for haemagglutinin and for viable virus (plaque count) (Table 4). The results of this experiment show that infection produces a substantial (39 %) increase in the amount of RNA

Table 3. *Effect of encephalomyocarditis-virus infection on the net amounts of nuclear and mitochondrial ribonucleic acids of Krebs II cells*

Suspensions of ascites-tumour cells (2×10^7 cells/ml.; 10 ml./flask) were incubated with encephalomyocarditis virus (3 plaque-forming units of virus/cell) for 2, 4 and 6·5 hr., together with uninfected controls. The flasks were removed, and the cells washed and disrupted by the double-osmotic-shock method. The lysates were fractionated to yield nuclear, mitochondrial and microsome-plus-cell-sap fractions (Martin, Malec, Coote & Work, 1961*a*). The mitochondrial fraction was analysed for protein and RNA content, and DNA and RNA estimates were carried out on the nuclear fraction. All results have been calculated as the total amount of each constituent for 10^8 cells, assuming the distribution of protein and DNA to be that given in Table 5 [Martin *et al.* (1961*a*)]. The total nuclear-plus-cytoplasmic RNA content/10^8 cells was 230 μg. of RNA phosphorus.

		Period of infection (hr.)		
		2	4	6·5
		RNA P (μg./10^8 cells)		
Cell fraction	Flask			
Mitochondria	Infected	20·6	25·7	30·8
	Control	22·5	20·7	22·2
	Difference	−1·9	+5·0	+8·6
Nuclei	Infected	25·8	24·3	27·9
	Control	25·1	29·8	32·4
	Difference	+0·7	−5·5	−4·5
Net change in RNA P (% of total cell RNA P)		−0·5	−0·2	+1·8

Table 4. *Distribution of viral haemagglutinin and infective virus among cytoplasmic fractions from infected Krebs II ascites-tumour cells*

Portions of the mitochondrial and mixed microsome-plus-cell-sap fractions from the experiment described in Table 3 were examined for their virus content by haemagglutinin titration and infective particle (plaque) count assay. Results are expressed as haemagglutinin units or plaque-forming units/10^8 cells.

		Period of infection (hr.)		
Cell fraction	Virus assay method	2	4	6·5
Mitochondria	Haemagglutinin	0	150	12300
	10^{-6} × Infective particles	1·75	15·5	650
Microsomes plus cell sap	Haemagglutinin	12	12	675
	10^{-6} × Infective particles	0·4	1·0	22

in the mitochondrial fraction 6·5 hr. after infection and that this is almost balanced by a corresponding decrease in the amount of nuclear RNA so that the overall change is negligible (1·8 %). In a second similar experiment the percentage increase in mitochondrial RNA in infected cells at 6 hr. was even greater than that shown in Table 3.

DISCUSSION

Infection caused profound alterations in the RNA metabolism of the tumour cell. Since there was a slow fall in the rate of metabolism of the control cells throughout the course of the incubation, the results for the infected cells obtained in the experiment described in Table 2 have been expressed as a percentage of the corresponding control and plotted in Fig. 1, together with the figures for virus haemagglutinin titre in the mitochondrial fraction. It is evident that coincident with the appearance of virus in the mitochondrial fraction there is an enormous increase (320 %) in the rate of orotic acid incorporation into the RNA of this fraction. At the same time there is a similar, though smaller, increase in the rate of turnover of RNA in the microsomal fraction. A large increase in total cell RNA turnover at about the time of synthesis of complete virus was reported by Martin *et al.* (1961*b*). The present results indicate that this is accounted for largely by the increase in turnover of the RNA of the mitochondrial and microsomal fractions.

It has been proposed (Martin & Work, 1961) that the synthesis of RNA in ascites-tumour cells takes place entirely in the nucleus. This contention is supported by the results given in Table 2, which show a constant relationship between the rates of orotic acid incorporation into the ribonucleic acids of the nuclear and cytoplasmic fractions, in both normal cells and cells up to 4 hr. after infection with encephalomyocarditis virus. However, in cells infected for a greater period, the rate of cyto-

plasmic RNA synthesis, calculated from the ratios of specific activities of the uridylic acid in RNA and the acid-soluble pool (Martin *et al.* 1961*b*), far exceeds that which would be expected from the nuclear RNA turnover rate, assuming that the nucleo-cytoplasmic relationship had remained unaltered. Therefore it may be argued that this difference represents the synthesis of viral RNA. However, when the amount of this anomalous, newly synthesized cytoplasmic RNA is compared with the amount of virus-associated RNA expected to be formed during the same 30 min. period, as estimated from the increase in haemagglutinin titre by the data of Faulkner *et al.* (1961), the virus-associated RNA represented only 5–8% of the total RNA formed. Hence, the stimulation in mitochondrial and microsomal RNA turnover in the later stages of infection cannot be accounted for in terms of synthesis of virus-associated RNA.

From the beginning of infection the rate of precursor incorporation into the RNA of the nucleus was progressively inhibited (Fig. 1). As nearly 90% of the total cell RNA turnover takes place in the nucleus, and as it is probable that most or all of the cell's RNA is synthesized at this site, it is likely that the marked inhibition of RNA turnover in the whole cell (Martin *et al.* 1961*b*) and the slight inhibition of turnover in the cytoplasmic constituents in the eclipse phase (Table 2; Fig. 1) can be ascribed to the effect of infection on nuclear RNA synthesis. Although this inhibition is doubtless a consequence of viral infection, there is little evidence to suggest that it is concerned with the replication of viral constituents.

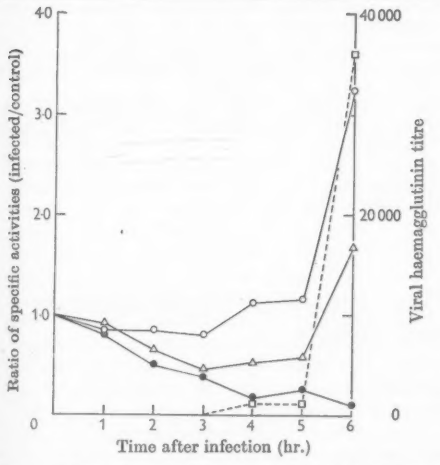

Fig. 1. Effect of viral infection on rate of [6-^{14}C]orotic acid incorporation into the RNA of nuclei, mitochondria and microsomes. Estimates of specific radioactivities of the RNA from the nuclei, mitochondria and microsomes of virus-infected Krebs II cells have been plotted as a ratio of the estimates of specific radioactivity of control uninfected cells. Experimental details are given in Table 2. ●, Nuclear RNA; ○, mitochondrial RNA; △, microsomal RNA; □, haemagglutinin titre (units/10^8 cells) of the mitochondrial fraction.

Sanders, Huppert & Hoskins (1958) and Sanders (1960) have shown that the ability of encephalomyocarditis virus to kill Krebs II cells is independent of its power to multiply within them, and a similar separation between cell-killing properties and viral replication has also been observed in HeLa cells infected with poliomyelitis virus (Ackermann, Rabson & Kurtz, 1954). Hence it is possible that the inhibition of nuclear RNA synthesis is associated with the process, caused by infection but unrelated to the replication of viral RNA or protein, which leads to the death of the cell.

Huppert & Sanders (1958) showed that infective RNA could be extracted by cold aqueous phenol from Krebs II ascites-tumour cells that had been infected with encephalomyocarditis virus, although no RNA could be obtained from the virus particles by this treatment. Bellett & Burness (1960) have used the osmotic-shock method of Martin *et al.* (1961*a*) to localize the formation of infective RNA, and found it to be almost entirely confined to the nucleus during the first 4·5 hr. after infection, thus giving a direct demonstration that the nucleus is the site of infective RNA synthesis in this system.

Bellett & Burness (1960) found that the titre of infective RNA in the nucleus decreased sharply after 4·5 hr., and that this loss was accompanied by a rise in the infectivity of the RNA from the mitochondrial fraction. These results suggested a nucleo-cytoplasmic transfer of the viral RNA. The present results (Table 3) strongly support the idea of a transfer of RNA from nucleus to cytoplasm, but the amount of RNA transferred is much greater than would be required for the formation of virus particles. It may well be, however, that the cell produces considerably more virus-specific RNA than it can incorporate into virus. The appearance of free infective RNA in the culture medium at the end of the virus growth cycle suggests that this does in fact occur (Huppert & Sanders, 1958).

Although the present results demonstrate unequivocally that virus-protein synthesis takes place in the cytoplasm of the Krebs tumour cell, they are less definitive with regard to the site of RNA synthesis. We have emphasized elsewhere (Work, 1960; Martin & Work, 1961) that biological replication always requires the simultaneous presence of DNA and RNA and that neither DNA

nor RNA virus is capable of replication except in an environment that can supply the missing components. Such a requirement could well explain the apparent formation of infective RNA within the nucleus of the ascites-tumour cell and the transfer of this to the cytoplasm before synthesis of complete virus can begin.

SUMMARY

1. The effect of infection with encephalomyocarditis virus on the rate of incorporation of ^{14}C-labelled precursors into the protein and ribonucleic acid of the subcellular components of Krebs II ascites-tumour cells has been investigated.

2. The nucleus, which was the major site of ribonucleic acid synthesis in the cell, contained negligible amounts of virus. Infection caused a marked progressive inhibition of orotic acid incorporation into nuclear ribonucleic acid, and also some net loss of ribonucleic acid from the nucleus. It is suggested that this disruption of nuclear ribonucleic acid metabolism is related to the cell-killing properties of the virus.

3. Most of the virus sedimented with the mitochondrial fraction. The amount of mitochondrial ribonucleic acid increased progressively during infection by an amount approximately equivalent to that lost from the nucleus. Incorporation of orotic acid into mitochondrial ribonucleic acid was slightly inhibited for the first 3 hr. after infection; thereafter it was stimulated, reaching 320% of the control at 6 hr.

4. Less virus appeared in the microsomal fraction. The pattern of incorporation into microsomal ribonucleic acid was similar to that of the mitochondria, but less pronounced. In both fractions, the increase in incorporation rate was apparently related to the amount of virus present, but estimates showed that only 5–8% of this newly synthesized ribonucleic acid could be ascribed to viral ribonucleic acid formation.

5. In all cytoplasmic fractions, infection caused an initial slight stimulation of valine incorporation into protein, which was most marked in the cell-sap fraction. This was followed by a period of moderate inhibition, until appreciable amounts of virus had accumulated intracellularly, when incorporation into mitochondrial protein was again elevated.

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A Study of the Kinetics of the Fibrillar Adenosine Triphosphatase of Rabbit Skeletal Muscle

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One of the most puzzling features of the kinetics of the adenosine-triphosphatase activity of actomyosin and of the myofibrils in which it is contained is the so-called explosive phase of hydrolysis which occurs immediately after addition of substrate and which is followed under certain special conditions by a 'linear' phase of lower, but constant, velocity. These features were originally studied by Weber & Hasselbach (1954) in myofibrillar preparations at low ionic strengths ($< 0{\cdot}15$), but later Tonomura & Kitagawa (1957) showed that they were also characteristic of the hydrolysis of adenosine triphosphate by myosin B in the presence of Ca^{2+} ions, at high ionic strength ($> 0{\cdot}5$). Tonomura & Kitagawa (1960) have extended their observations on myosin B to include

the effect of other bivalent ions, such as Mg^{2+}, Mn^{2+} and Sn^{2+}, and of chelating agents such as ethylenediaminetetra-acetate, and have shown that both prolonged dialysis and treatment of myosin B with very low concentrations of ethylenediaminetetra-acetate ($< 10\,\mu$M) abolish the explosive phase almost completely, and that it can be restored by addition of small amounts of Mg^{2+} but not of Ca^{2+} ions.

In some respects the present study of the myofibrillar adenosine triphosphatase at lower ionic strengths (0·04–0·25) confirms the findings of Tonomura & Kitagawa, particularly the observation that the explosive phase is more prominent in the presence of Mg^{2+} ions than in their absence, even though Ca^{2+} ions may also be present in large amounts. Nevertheless, the extent and duration of this phase, with fibrils at low ionic strength, is further complicated under most conditions by pronounced inhibition by the products, so that a true stationary phase very rarely follows the so-called explosive phase, but rather a phase of steadily falling velocity. It so happens that the special conditions under which the reaction has usually been studied, that is at an ionic strength of ~0·15 and at temperatures between 0° and 20° with magnesium as activator (cf. Weber & Hasselbach, 1954), are precisely those in which the phase succeeding the explosion is truly stationary and unaffected by addition of the products.

The question of product inhibition is one that seems to have been overlooked in most studies, although Kalckar (1944) and Green & Mommaerts (1954), using Ca^{2+} ions as activator at pH 6·4, have clearly demonstrated the inhibition of myosin adenosine triphosphatase by adenosine diphosphate. The myofibrillar enzyme shows very similar inhibition by the products under a wide range of conditions, e.g. with Ca^{2+} ions as activator, at all ionic strengths between 0·04 and 0·25 and at all temperatures from 0° to 35°, and with Mg^{2+} ions as activator, at all ionic strengths up to 0·25 at 35°, but only at the lowest ionic strength at 0° and 18°. From this it is clear that many false deductions can be made from progress curves of hydrolysis unless the effect of the products is taken into consideration, particularly in relation to the explosive phase. For example, the 'exponential' time curve, characteristic of product inhibition, can lead to the belief that a marked and extensive explosive phase is present, when in fact most of the effect is due to the gradual accumulation of products. Even in the presence of Mg^{2+} ions, which favour the explosive initial phase, it is much less extensive than Weber & Hasselbach (1954) originally thought, and with Ca^{2+} ions as the sole activator it either does not exist or is of such short duration that it cannot be distinguished from small, but inevitable, errors of estimation in the early stages.

METHODS

Abbreviation. P_i, inorganic orthophosphate.

Reagents. All reagents were of AnalaR grade (British Drug Houses Ltd. or L. Light and Co. Ltd.), except ATP and ADP, which were of the highest grade from Sigma Chemical Co. All reagents were treated by passage through a bed of the ion-exchange resin Dowex 50 × 8 (H^+ form), to remove traces of heavy metals, and, with the exception of $CaCl_2$, the reagents contained less than 1 p.p.m. of Ca. The water used was passed through a mixed-resin bed (Elgastat), and had an electrical resistance $> 2 \times 10^6\ \Omega$/cm³.

Preparation of glycerol-treated fibrils. Longissimus dorsi and psoas muscles (100 g.) from a freshly killed rabbit were homogenized in a small, high-speed homogenizer in lots of 2 g. in 7 ml. of ice-cold buffer (20 mM-imidazole, 80 mM-KCl and 5 mM-potassium oxalate, pH 7·2). Finally the volume of pooled homogenate was made up to 1 l. and stored at 0° overnight. Next day it was filtered through washed butter-muslin to remove fibre fragments and debris, and centrifuged at −1° at 2300*g* for 30 min. The supernatant was discarded and the fibrils were washed three times, by centrifuging and decantation at 0°, in a buffer consisting of 20 mM-imidazole, 80 mM-KCl, 5 mM-potassium oxalate and 4·1 M-glycerol. Finally, the fibrils were suspended in a volume of 1 l. of the glycerol buffer and stored at −9°. Prepared in this way, the fibrils are of uniform size and free of fibres and fragments of connective tissue. The method was originally due to Webster (1953). For use in enzymic studies portions of the fibril suspension were removed, and washed four times at 0° with the buffer solution appropriate to the experiment.

Determination of nitrogen content of fibrils. A portion (20 ml.) of fibril suspension (≡ 2 g. of muscle) was washed free of glycerol with 100 mM-KCl (three washes) and then made up to a final volume of 20 ml. in the latter solution. Portions (1 ml.) were digested, for 1 hr. after clearing, in 2 ml. of conc. H_2SO_4 + 80 mg. of a mixture of Se (1 g.) and K_2SO_4 (32·5 g.). The digest was made up to 100 ml. with water, and 1 ml. portions were removed for estimation of N content by direct nesslerization (1 ml. portion + 15 ml. of water + 2 ml. of Nessler's reagent + water to 25 ml.). The colour was measured in 1 cm. cells in a Unicam spectrophotometer (SP. 600) at 420 mμ, a digested reagent blank being used. The scatter of N values on five portions of the same fibril suspension was within 0·5% of the average value. The average N contents of the five glycerol-treated preparations used in this study were respectively 19·7, 19·3, 18·4, 18·4 and 18·9 mg. of N/g. of original muscle. The value given by Helander (1957) for myofibrillar protein N, free of stroma and connective tissue, is approx. 18·0 mg. of N/g. of fresh muscle.

Composition of buffer and substrate solutions, and their effect on ionic strength. The buffer used throughout was 40 mM-imidazole (p*K* 7·2) at pH 7·20 (final), which contributes 0·02 to the ionic strength (*I*). In the magnesium system the initial substrate (ATP) concentration in the control experiments was 4 mM and the $MgCl_2$ concentration 4 mM; 0·2 mM-$CaCl_2$ was also added to inhibit any traces of relaxing factor which might be present (cf. Bendall, 1953). From the formation of the complex ion, $(ATP–Mg)^{2-}$, calculated according to the stability constant of ~ 3000 M^{-1} given by Smith & Alberty (1956), this mixture contributes

$\sim$ 0·022 to I. The remainder of I, when $> 0{\cdot}042$, was made up by addition of KCl. In the calcium system, the initial ATP concentration was 4 mM and the $CaCl_2$ concentration 8 mM in the control experiments. These together contribute approx. 0·024 to I (cf. Smith & Alberty, 1956). The concentration of activator and substrate in both systems is in the optimum range given by the results of Perry & Grey (1956) and Weber (1959). Such substrate concentrations must be far above the Michaelis constant, K_s, of the reaction, since it was found in the present work that the rates of hydrolysis were still maximal, even at 1 mM-substrate, in either system.

Preparation of myokinase. Myokinase was prepared by the method of Kalckar (1943) but was not purified beyond the stage of the crude preparation employed by Bendall (1954). In the experiments cited it was added to the reaction mixture to give a final concentration of 0·04% of the crude protein. This concentration is twice that necessary to give the maximal rate of appearance of P_i, when added together with myofibrils to a mixture of 4 mM-ADP + 4 mM-$MgCl_2$ at I 0·15, and 35°.

Measurement of rate of hydrolysis. Portions of washed fibrils were pipetted into a 100 ml. beaker, held in a constant-temperature water bath, and provided with a mechanical stirrer. The buffer and activating ion were then added and finally the reactant solution, with vigorous stirring (final volume 72 ml.). The reactant was added in a volume of 4 or 6 ml. from a rapid-delivery pipette in $< 0{\cdot}5$ sec. Immediately after this addition portions of about 4 ml. were withdrawn in turn as quickly as possible, and each was dropped into 12 ml. of ice-cold 6% trichloroacetic acid contained in weighed flasks and stirred vigorously with a magnetic stirrer. The size of the samples was determined by reweighing the flasks. The method, with two operators, one pipetting and the other timing, allows withdrawal of a sample within 3·5–5 sec. of adding the reactant, and then every 4 or 5 sec. thereafter. The error in timing is less than 0·25 sec.

The P_i formed was estimated by the method of Allen (1940), appropriate enzyme and reagent blanks being included. The blank samples were put through exactly the same processes as the other samples, except that ATP was added to them after adding trichloroacetic acid to the fibril suspensions. The greatest care is necessary in the preparation of such blanks, particularly in view of the results of Marsh (1959), who found the blank value to vary independently and irregularly with the amount of ATP present. No such anomalies appeared in the present work, but, on the contrary, the blank value due to the small impurity of P_i in the ATP was always directly dependent on the amount of ATP added, even at high ATP concentrations. However, if the NaOH used to neutralize the trichloroacetic acid extracts had been stored in glass, rather than in polythene bottles, the blank values became anomalous, probably owing to interference with the colour development by complex silicates, leached from the glass.

RESULTS

The conditions for the control experiments have already been described under Methods. In experiments on addition of products, the concentration of activating ions (Ca^{2+} or Mg^{2+}) was kept in the same proportion to the combined concentrations of ATP + ADP as in the control experiments, i.e. 4 mM above this concentration in the calcium system and equal to it in the magnesium system. In these respects the experiments differ entirely from those of Perry & Grey (1956) on the magnesium system, where ADP was added in excess to a system containing equal concentrations of $MgCl_2$ and ATP. The units employed throughout to express the amounts of substrate or products initially present (a, Y) or of products formed (x) are μmoles of substrate or products/mg. of fibrillar N. The rates of hydrolysis are expressed as units of ATP hydrolysed to ADP/sec. ($\equiv$ units of P_i formed/sec.).

Nature of the progress curves for hydrolysis

The progress curves can be broadly divided into two types: (a) those which show a short explosive phase followed by a 'linear' phase of steady-state velocity (V_m), and (b) those which are curvilinear throughout. The two types are best illustrated from results with the magnesium system at 18·5°, where we see from Figs. 1 (a) and 1 (b) that a mere decrease of I from 0·15 to 0·042 changes the curve from type (a) to type (b). Besides the features described above, the type (a) curve is further characterized by the absence of inhibition by either of the products ADP or P_i, and, by the corollary of this, that addition of myokinase to remove ADP only slightly affects the shape of the curve, as the Figure shows. This type of curve is found only in the magnesium system where it is restricted to values of I from 0·15 to 0·25 and to temperatures between 0° and 20° (see Table 2 below). It does not occur at all at 35°, where all the curves are of type (b), from $I = 0{\cdot}04$ to $I = 0{\cdot}25$. Curves of type (b), besides being curvilinear throughout their course, differ from type (a) in showing no well-defined explosive phase in plots of x against t; in being more or less severely inhibited by addition of the products [see Fig. 1 (b)] and in being markedly affected by the addition of myokinase, which, by removing the main inhibitor, ADP, increases the rate of hydrolysis in the later stages. This latter effect is illustrated in Fig. 2 for the magnesium system at 35° and I 0·15. Curves of this type, as we have said, are found in the magnesium system at 35° and at low I at 0° and 18·5°, but are characteristic of the calcium system at all the values of I and temperatures studied (cf. Tables 1 and 2 below).

It follows from these results that plots of the rates of hydrolysis per unit volume against the enzyme concentration should give straight lines for curves of type (a), and curves indicating deceleration for those of type (b). This is illustrated in Fig. 3 for the magnesium system. The type (a) curve at

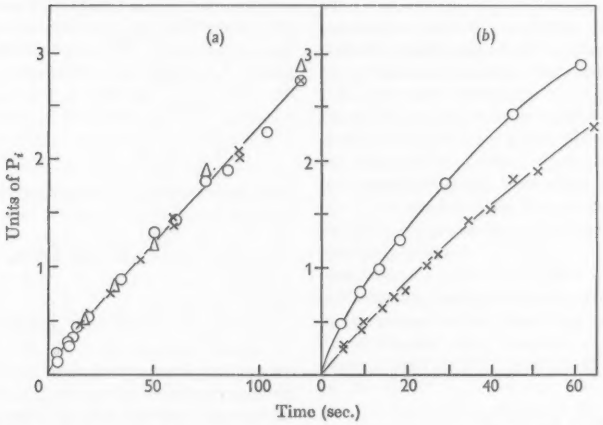

Fig. 1. Two types of progress curve found in the magnesium system, and the effect on them of adding either the product (ADP) or myokinase to remove it. Included in this and all other experiments with the magnesium system is 0·2 mM-$CaCl_2$. Temp., 18·5°; 4 mM-ATP + 4 mM-$MgCl_2$ for control, and 4 mM-ATP + 2 mM-ADP + 6 mM-$MgCl_2$ for product curve; initial substrate in both cases was 6·30 μmoles of ATP/mg. of fibrillar N. Crude myokinase was added where noted to give a final concentration of 0·04% of the crude protein. Curve (*a*) at *I* 0·15; curve (*b*) at *I* 0·042. ○, Control; △, control + myokinase; ×, control + ADP.

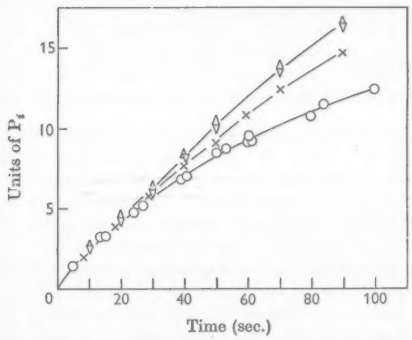

Fig. 2. Effect of myokinase on curves of type (*b*). Temp., 35°; *I* 0·15; 4 mM-ATP + 4 mM-$MgCl_2$; initial substrate, 25 moles of ATP/mg. of fibrillar N. Myokinase was added as in Fig. 1. For the observed values in the two lower curves: ○, control; ×, control + myokinase. For the calculated $V_m t$ values [see equations 9(*a*) and 9(*b*)]: △, control; ▽, control + myokinase.

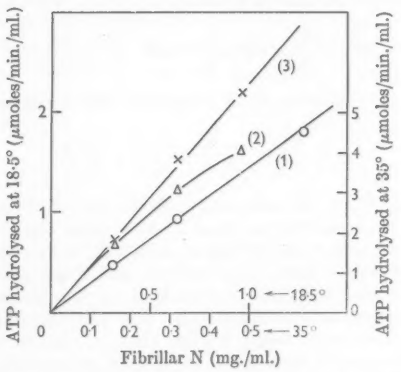

Fig. 3. Dependence of the steady velocity of hydrolysis (V_m) on the enzyme concentration for curves of types (*a*) and (*b*) in the magnesium system. Curve 1: *I* 0·15; temp., 18·5°; curves 2 and 3: *I* 0·042; temp., 35°. Velocities in curve 2 are average values of $\Delta x/\Delta t$ for first 30 sec. of reaction; velocities in curve 3 are calculated from equation (2) for the first 50 sec. of the reaction. Concentration of ATP 4 mM, and $MgCl_2$ 4 mM, throughout. Six points on the time curves were used to calculate each velocity.

18·5° is seen to be very nearly linear, whereas the type (*b*) curve at 35°, when expressed in terms of average rates for the first 30 sec. of the reaction, bends towards the abscissa, as expected. When, however, the rates are calculated from V_m values derived from the appropriate equation for product inhibition (see next section), they become directly proportional to enzyme concentration.

Theoretical analysis of the curves

Type (a). Curves of type (*a*) can be analysed simply by extrapolating the linear portion back to the time base, which gives one intercept, *X*, on the

product axis, which is generally positive, and one, t_s, on the time axis, which is generally negative. The stationary velocity of the linear phase is called V_m, by analogy with the calculated velocity of type (*b*) curves. The true initial velocity, V_o, cannot be estimated even approximately in most cases, because the initial rapid portion of the curve often lasts less than 5 sec., which is the earliest time at which a sample can be withdrawn for estimation by the method described here.

Type (b). Curves of this type are more difficult to analyse than those of type (*a*), because they are not only modified by addition of the products, but may contain a hidden explosive phase as well. In general, however, we can make use of an equation analogous to the integrated rate equation of Foster & Niemann (1955) derived from the reaction steps:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} E \begin{matrix} \nearrow P_1 \\ \searrow P_2 \end{matrix}$$

$$EP_1 \underset{k_6}{\overset{k_5}{\rightleftarrows}} E+P_1, \quad EP_2 \underset{k_8}{\overset{k_7}{\rightleftarrows}} E+P_2,$$

where E is concentration of enzyme, S of substrate, P_1 of ADP and P_2 of P_i. Making the assumption that $k_3 \gg k_4$, and putting

$$K_s = \frac{k_2}{k_1}, \quad K_1 = \frac{k_5}{k_6} \quad \text{and} \quad K_2 = \frac{k_7}{k_8},$$

the rate equation to be integrated then becomes

$$-V_m \frac{dt}{dS} = 1+\frac{K_s}{S}+\frac{K_s P_1}{K_1 S}+\frac{K_s P_2}{K_2 S}, \qquad (1)$$

where V_m is the maximum velocity and S, P_1 and P_2 are measured at time t. Now from our own results and those of Weber (1959), K_s is less than 0·1 mM for both the calcium- and magnesium-activated reactions, whereas S in the present experiments was initially $\geqslant 4$ mM and was never allowed to fall below 1·5 mM. Hence, even in the late stages of the reaction the term K_s/S was always less than 0·07, whereas the term $-V_m(dt/dS)$ had by that stage generally reached values in excess of 2·0. It follows that K_s/S can be ignored as a first approximation, and we may then integrate as follows:

$$V_m t' = (1-K')\, x + K' a \ln \frac{a}{a-x}, \qquad (2)$$

where a represents the initial amount of substrate/mg. of fibrillar N, x the amount of either product formed at time t (sec.), V_m the μmoles of ATP hydrolysed/mg. of fibrillar N/sec., K' equals $K_s/K_1+K_s/K_2$ and t' equals $t-t_s$.

Similar equations can be derived for the cases where both products are added initially in equal amount Y, or one product is added in amount y. For simplification, let a, $x=a_1$, x_1, and $a_1/(a_1-x_1)=\alpha$ for the experiment with substrate alone. Similarly, when products are added initially, let a, $x=a_2$, x_2 and $a_2/(a_2-x_2)=\beta$. Then for addition of both products in amount Y, we have

$$V_m t' = (1-K')\, x_2 + K'(a_2+Y) \ln \beta. \qquad (3)$$

But, for addition of either product separately in amount y:

$$V_m t' = (1-K')\, x_2 + \left(K' a_2 + \frac{K_s}{K_1} y_1\right) \ln \beta \qquad (4a)$$

$$= (1-K')\, x_2 + \left(K' a_2 + \frac{K_s}{K_2} y_2\right) \ln \beta. \qquad (4b)$$

In any of these cases, however, there may be a hidden explosive phase in the initial stages, not taken into account by the equations. In such a case, t' is equivalent to $t-t_s$, where t_s is the negative intercept on the time axis of the linear curve derived from the appropriate equation. In practice, t_s can be eliminated by comparing the progress curves for substrate alone and substrate + products at fixed times from the start. It then follows from equations (2) and (3) that at time t

$$V_m t' = x_1 + K'(a_1 \ln \alpha - x_1)$$
$$= x_2 + K'[(a_2+Y) \ln \beta - x_2]. \qquad (5)$$

Therefore

$$x_1 - x_2 = K'[\{(a_2+Y) \ln \beta - x_2\} - (a_1 \ln \alpha - x_1)]. \qquad (6)$$

We know all the terms except K', which is given by plotting a series of values of x_1-x_2 against the corresponding differences of the factors of K', and V_m which is then evaluated by plotting the two equivalent right-hand terms of equation (5) against t. Both sets of points should then lie on one line with slope V_m and a negative intercept on the time axis of t_s. An alternative method, useful if V_m does not change during the course of the reaction, is to take the times required in each case for a given amount of products, x, to be formed.

Then:

$$t'_1 - t'_2 = t_1 - t_2$$
$$= \frac{K'}{V_m}[(a_1 \ln \alpha - x) - \{(a_2+Y) \ln \beta - x\}]. \qquad (7)$$

From this we can evaluate K'/V_m. V_m can then be evaluated from

$$V_m = \frac{dx}{d\left[t_1 - \frac{K'}{V_m}(a_1 \ln \alpha - x)\right]}$$
$$= \frac{dx}{d\left[t_2 - \frac{K'}{V_m}\{(a_2+Y) \ln \beta - x\}\right]}. \qquad (7')$$

Once we have evaluated V_m and K' in this way it is a simple matter to calculate K_s/K_1 and K_s/K_2 by means of equations (4*a*) and (4*b*) from the appropriate experiments with each product added separately.

Analysis of curves showing product inhibition

Calcium system. The above analysis applies most satisfactorily to the calcium system, where the explosive phase either does not exist or is of very short duration. The results at 18·5° provide an excellent illustration because we have examples, at I 0·042, 0·15 and 0·25, of three types of product equation, where $K' > 1{\cdot}0$, $K' = 1{\cdot}0$ and $K' < 1{\cdot}0$ respectively. Moreover, we have experimental verification at I 0·15 that K_m is very small in comparison with S, because the velocity, V_m, was found to be constant throughout a whole range of S values from 1 to 8 mM, when the initial amount of substrate per unit of enzyme was held constant. Fig. 4 shows the plots of $\ln [a/(a-x)]$ against t for the three cases where the concentrations of products and substrate, when these were initially added together, were so arranged that $a_1 \equiv a_2 + Y$. It will be noted that in the first case, where I is 0·042, the curves for the control and for the products both bend towards the time axis, and the latter is below the former, characteristic when $K' > 1{\cdot}0$, whereas in the second case, at I 0·15, both curves are rectilinear and coincident, characteristic when $K' = 1{\cdot}0$, and in the third case, at I 0·25, both curves bend away from the time axis, and the product curve is above the control, characteristic when $K' < 1{\cdot}0$. Analysis, by means of equations (6) and (7), showed that K' had a value of ~1·9 at I 0·042, of 1·0 at I 0·15, and of ~0·7 at I 0·25. The reliability of this analysis can be checked by plotting the values of $V_m t'$, calculated from the experimental points by equation (3), against t for the control and for the products in each case. This is done in Fig. 5, where all the curves are now rectilinear, and the experimental points lie very close to the curve in each case. Also all the curves pass very nearly through the origin, so that little or no explosive phase can have been present. This point is discussed in more detail below.

Once the relative inhibition constant for both products, K', was known, those for each product were evaluated separately from equations (4*a*) and (4*b*). In the present example of the calcium system at 18·5° such analysis showed that ADP was the more effective inhibitor at I 0·042 and I 0·15, where P_i contributed only about a third of the total inhibition, whereas at I 0·25 the inhibition was distributed equally between the two products. Similar results were obtained at 35°, as seen from Table 1, which summarizes all the results for this system at the three temperatures 0°, 18·5° and 35° and the three values of I 0·042, 0·15

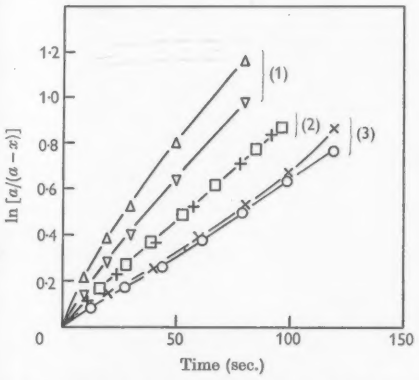

Fig. 4. Calcium system at 18·5°. The effect of plotting $\ln [a/(a-x)]$ against t at three values of I. For control curves: ATP, 4 mM; $CaCl_2$, 8 mM. For product curves: ATP, 3 mM; ADP, 1 mM; P_i, 1 mM; $CaCl_2$, 8 mM. The following pairs of curves are shown: Curve (1): I, 0·042; △, control; ▽, with products. Curve (2): I, 0·15; □, control; +, with products. Curve (3): I, 0·25; ○, control; ×, with products. *Note*: values for curve (1) have been multiplied by 2 to distinguish them from those for curve (2).

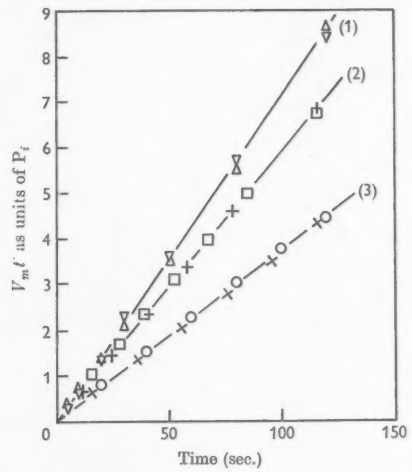

Fig. 5. Calcium system at 18·5°. The curves of Fig. 4 are expressed as $V_m t'$ against t calculated from equation (4). Conditions and symbols are as in Fig. 4. Initial substrate, a, in control curves 6·30, and in product curves 4·90 μmoles of ATP/mg. of fibrillar N. *Note*: $V_m t'$ values in curve (1) have been multiplied by 1·25 to distinguish them from those for curve (2).

Table 1. *Relative product inhibition constants for the calcium system at three temperatures and ionic strengths, with their standard errors*

Degrees of freedom are given in parentheses.

Temp.	I	K'	K_s/K_{ADP}	K_s/K_{P_i}
1·0°	0·042	1·00 (6)	1·00±0·001 (8)	0*
	0·150	0·83±0·004 (5)	0·52±0·025 (8)	0·31*
	0·250	0·86±0·003 (4)	0·46±0·04 (6)	0·40*
18·5	0·042	1·91±0·011 (5)	1·22±0·02 (6)	0·60±0·01 (5)
	0·150	1·00 (16)	0·63±0·04 (7)	0·34±0·04 (5)
	0·250	0·70±0·01 (8)	0·34±0·015 (6)	0·34±0·06 (6)
35	0·042	2·20±0·03 (6)	1·50±0·06 (7)	0·54±0·04 (8)
	0·150	1·38±0·046 (6)	1·34±0·026 (7)	0·16±0·016 (8)
	0·250	0·67±0·006 (15)	0·57±0·04 (12)	0·13±0·06 (13)

* By difference.

and 0·25. At 1·0°, unlike the higher temperatures, K' does not change much as I is increased, but remains close to 1·0 throughout, just as in the magnesium system at 0° it is always close to zero (cf. Table 2).

Magnesium system. The results of the studies of product inhibition in the magnesium system are more difficult to analyse than those for the calcium system, first because there is some evidence of a very early explosive phase, and secondly because a sudden decrease in slope appears after about 40 sec. in plots of $V_m t'$ against t. Analysis of this phenomenon at 35° is fortunately simplified at the lowest strength (I 0·042) because K' turns out to be unity, so that, following equation (5):

$$V_m t' = a_1 \ln \frac{a_1}{a_1 - x_1} \text{ for control}$$

$$= (a_2 + Y) \ln \frac{a_2}{a_2 - x_2} \text{ for initial addition of products.} \quad (8)$$

Furthermore, under the above conditions of I and temperature only ADP inhibits and not P_i, as shown in Fig. 6, where $V_m t'$, calculated from the experimental points by equation (8), is plotted against t for substrate only, substrate + ADP and substrate + ADP + P_i. The points from all these experiments lie closely on the curve, which has a sharp inflexion at ~40 sec. In this case, as in most others of this type, $V_{m.1}$ (up to 40 sec.) is ~1·4 times $V_{m.2}$ (from 40 to 150 sec.). Also the first straight line, $V_{m.1} t'$ against t, does not pass exactly through the origin, but cuts the axis at ~ -2 sec. ($= t_{s.1}$). This early non-linear phase, which lasts about 5 sec., is evidently the anomalous explosive phase of Weber & Hasselbach (1954). It will be discussed in detail below. Very similar diphasic curves are obtained at I 0·15 and I 0·25 at 35°, and at I 0·042 at 18·5° (Tables 2 and 3), although K' varies widely, being ~1·3, ~0·2 and ~0·5 respectively. In spite of these differences, however, $V_{m.1}/V_{m.2}$ lies between 1·24 and 1·50 in all cases (see Table 3). Also Table 2 shows that P_i acts as an inhibitor only at I 0·042 and 18·5° and at I 0·15 and 35°, another marked point of difference from the calcium system where such inhibition evidently occurs in all cases.

Effect of myokinase in relieving the inhibition by the adenosine diphosphate formed. It is of some interest to consider the kinetics of the reaction when myokinase is present, as in the experiment illustrated in Fig. 2 for the magnesium system at I 0·15 and 35°. The reactions now taking place can be written, according to Bendall & Davey (1957), as

$$2\text{ATP} \xrightarrow[\text{triphosphatase}]{\text{adenosine}} 2\text{ADP} + 2\text{P}_i, \quad \text{(i)}$$

$$2\text{ADP} \xrightleftharpoons{\text{myokinase}} \text{ATP} + \text{AMP}, \quad \text{(ii)}$$

$$\text{AMP} \xrightarrow{\text{deaminase}} \text{IMP} + \text{NH}_3. \quad \text{(iii)}$$

The overall reaction is therefore

$$\text{ATP} \longrightarrow \text{IMP} + 2\text{P}_i + \text{NH}_3. \quad \text{(iv)}$$

The only proviso is that myokinase must remove ADP as soon as it is formed in reaction (i), which was ensured in this experiment by adding a large amount of the active enzyme. Since neither IMP nor NH_3 appears to inhibit the fibrillar adenosine triphosphatase, it follows that P_i is now the only inhibitor present, two molecules of it being produced for each ATP molecule destroyed. Thus the integrated rate equation analogous to equation (2) becomes

$$V_m t' = \left(1 - 2\frac{K_s}{K_2}\right) x + 4a \frac{K_s}{K_2} \ln \left(\frac{2a}{2a - x}\right), \quad (9)$$

where K_s/K_2 is the relative inhibition constant for P_i, x the amount of P_i formed at time, t, in μmoles/mg. of fibrillar N, and V_m the velocity of reaction (i).

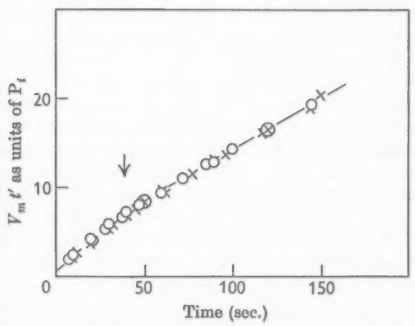

Fig. 6. Magnesium system at 35°. $V_m t'$ is calculated from equations (4) and (8) and is plotted against t for product inhibition at I 0·042, to illustrate the diphasic nature of the curves. Initial substrate, a, for control curves is 25·0, and for product curves, 18·8 moles/mg. of fibrillar N. ○, Two control experiments, nine points from each, at ATP 4 mM and $MgCl_2$ 4 mM; ×, effect of both products, or ADP alone, nine points from each experiment. Conditions for both products: ATP, 3 mM; ADP, 1 mM; P_i, 1 mM; $MgCl_2$, 4 mM. Conditions for ADP only: ATP, 3 mM; ADP, 1 mM; $MgCl_2$, 4 mM.

Inserting the known values of a (25 μmoles/mg. of N) and of K_s/K_2 (0·31), given in Fig. 2 and Table 2 respectively, this equation becomes:

$$V_m t' = 0{\cdot}38\left(x + 81{\cdot}5 \ln \frac{50}{50-x}\right). \qquad (9a)$$

Similarly the equation for the control curve shown in Fig. 2 becomes:

$$V_m t' = 0{\cdot}28\left(-x + 114 \ln \frac{25}{25-x}\right). \qquad (9b)$$

Application of these two very different rate equations to the actual results gives the two sets of points on either side of the top curve in Fig. 2. They agree fairly closely, and thus give added proof of the accuracy of the inhibition constants given in Table 1 for these particular conditions. Here again the curve has a characteristic inflexion at about 50 sec.

Summary of the effects of temperature and ionic strength on the two systems. To summarize the complicated results described in this section: (i) the $V_m t'$ curves in the magnesium system always tend to show two phases, in addition to a very short explosive phase, particularly in cases where the

Table 2. *Relative product inhibition constants for the magnesium system at three temperatures and ionic strengths, with their standard errors*

Degrees of freedom are given in parentheses.

Temp.	I	K'	K_s/K_{ADP}	K_s/K_{P_i}
0·6°	0·042	0·26±0·02 (6)	—	—
	0·150	0 (4)	0	0
	0·250	0 (4)	0	0
18·5	0·042	0·52±0·008 (8)	0·42±0·006 (7)	0·11±0·02 (6)
	0·150	0 (6)	0	0
	0·250	0 (4)	0	0
35	0·042	1·00 (8)	1·00 (8)	0 (8)
	0·150	1·28±0·013 (7)	0·93±0·024 (10)	0·31±0·06 (4)
	0·250	0·17±0·005 (7)	—	—

Table 3. *Steady velocities of splitting ($V_{m,1}$, $V_{m,2}$) and time intercepts ($t_{s,1}$, $t_{s,2}$) for the magnesium system, at three temperatures and ionic strengths*

Standard errors are given in parentheses.

Temp.	I	$10^4\ V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	Duration of phase with $V_{m,1}$	$10^4\ V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)	No. of expts.
0·6°	0·042	41·0 (1)	− 4 (7)	>240	*	*	4
	0·150	8·2 (1)	−17 (3)	~120	5·0 (0·5)	−100 (10)	2
	0·250	2·4 (0·2)	0 (1)	~240	1·6 (0·1)	−200 (50)	2
18·5	0·042	540 (20)	−2·3 (0·1)	45 (2)	430 (15)	−16 (1)	7
	0·150	230 (9)	−1·9 (0·5)	>240	*	*	9
	0·250	80 (20)	− 3 (0·5)	20 (5)	43 (5)	−22 (1)	2
35	0·042	1940 (20)	− 2 (0·2)	40 (2)	1190 (20)	−21 (1)	7
	0·150	2110 (30)	−1·4 (0·09)	38 (2)	1420 (10)	− 19 (1)	6
	0·250	890 (20)	0 (0·5)	25 (2)	660 (10)	− 14 (2)	2

* No detectable second phase.

products inhibit the reaction (cf. Tables 2 and 3), whereas in the calcium system there is no second phase and the early explosive phase is also absent or very restricted in extent (Table 4); (ii) the combined relative inhibition constants for both products (K') or for each product separately rise with increasing temperature and fall with increasing I in both systems, except in the calcium system at 1·0°, where K' is scarcely affected by I, and at I 0·25, where K' falls instead of rising with increasing temperature; (iii) the inhibition by the products, either separately or together, is always much greater in the calcium system than in the magnesium system, where indeed there is no inhibition at all at the lower temperatures and higher values of I (cf. Tables 1 and 2); (iv) increasing I has generally a far stronger inhibitory effect on hydrolysis in the magnesium system than in the calcium system, just as increasing temperature has a more marked accelerating effect (Tables 3 and 4). An exception to this rule occurs at 35°, where the velocity increases slightly in the magnesium system as I is raised from 0·04 to 0·15 and then decreases again at I 0·25, but the relative decrease is much less than at the lower temperatures. A similar increase in velocity as I is raised from 0·04 to 0·15 is a general feature of the calcium system.

Velocities of hydrolysis in mixed systems

As mentioned under Methods and as also shown by Bendall (1953) and Weber (1959), Ca^{2+} ions added in traces to the magnesium system have no effect on the shape of the progress curves, so long as all traces of relaxing factor have been removed in the process of washing. On the other hand, small amounts of Mg^{2+} ions added to the calcium system drastically modify the curves, and, in cases where the two systems are clearly distinguished, change them toward the pattern characteristic of the magnesium system, and away from that of the calcium system. This is particularly well illustrated at I 0·15 and 18·5°, by curves (1) and (4) in Fig. 7, where the curve for calcium alone (1) has almost double the velocity of that for a system to which 1 mM-$MgCl_2$ has also been added (4), and the latter has been modified to the 'linear' form characteristic of the magnesium system, instead of the 'exponential' form of the calcium system at this I. The effect is not so dramatic at I 0·042 and 18·5° [curves (2) and (3)], because both systems in any case give curves of exponential type under these conditions (cf. Tables 1 and 2), but K', calculated from an experiment in which the products were added, falls on addition of magnesium to the calcium system from the characteristically high value of 1·9 to 0·35, which is of the same order as K' for the magnesium system at this temperature (cf. Tables 1 and 2). At about 0°, the effect is even more striking, the stationary velocity of the calcium system being reduced by addition of $MgCl_2$ from $1{\cdot}5 \times 10^{-2}$ to $\sim 2{\cdot}3 \times 10^{-3}$ unit/sec. at I 0·042,

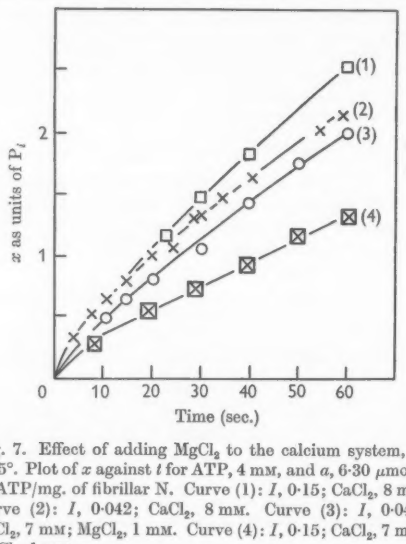

Fig. 7. Effect of adding $MgCl_2$ to the calcium system, at 18·5°. Plot of x against t for ATP, 4 mM, and a, 6·30 μmoles of ATP/mg. of fibrillar N. Curve (1): I, 0·15; $CaCl_2$, 8 mM. Curve (2): I, 0·042; $CaCl_2$, 8 mM. Curve (3): I, 0·042; $CaCl_2$, 7 mM; $MgCl_2$, 1 mM. Curve (4): I, 0·15; $CaCl_2$, 7 mM; $MgCl_2$, 1 mM.

Table 4. *Steady velocities of splitting (V_m) and time intercepts (t_s) for the calcium system*

Standard errors are given in parentheses.

Temp.	I	$10^4\ V_m$ (units/sec.)	t_s	No. of expts.
1·0°	0·042	151 (6)	−8·6 (1·3)	3
	0·150	171 (7)	−0·7 (0·7)	3
	0·250	128 (4)	−13·0 (2)	3
18·5	0·042	530 (13)	−0·7 (0·33)	10
	0·150	550 (5)	+0·12 (0·20)	8
	0·250	360 (12)	+0·50 (0·30)	4
35	0·042	1410 (25)	−0·50 (0·10)	7
	0·150	1680 (30)	−0·40 (0·30)	5
	0·250	1210 (40)	−1·00 (0·30)	6

Table 5. *Effect of pretreating fibrils at* 18·5° *with* 4 mM-*adenosine triphosphate–*4 mM-*magnesium chloride on the subsequent rate of splitting in the magnesium system*

Average values are for two experiments in each case.

		Control				Pretreated			
Temp.	I	$10^4\ V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	$10^4\ V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)	$10^4\ V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	$10^4\ V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)
18·5°	0·042	580	1	420	28	490	0·2	380	11
	0·150	230	3	*	*	230	0	*	*
35	0·042	1790	3	1290	20	1690	2	1340	11·0
	0·150	2100	1·4	1420	19	2000	0	1920	3·0

* No detectable second phase.

and from $1{\cdot}8 \times 10^{-2}$ to $\sim 6{\cdot}5 \times 10^{-4}$ unit/sec. at I 0·15, with a change in both cases from 'exponential' to 'linear' type (not shown).

Analysis of the so-called explosive phase and the effect upon it of pretreatment of the fibrils with adenosine triphosphate

In describing the kinetics of the hydrolysis reaction we have so far confined the discussion to the post-explosive phase, which we have shown to be inhibited more or less strongly by the products, ADP and P_i. It is now necessary to inquire whether indeed an explosive phase exists at all in the sense used by Weber & Hasselbach (1954), when these inhibitory effects are taken into account. This phase can best be described in terms of t_s, which for curves of type (*a*) is the intercept on the time axis of straight lines of best fit in plots of x against t, and for curves of type (*b*) is the intercept of plots of $V_m t'$ against t, where $V_m t'$ is calculated from the experimental points by means of the appropriate equation for product inhibition. Clearly t_s will be negative when an explosive phase exists, whereas in its absence it will be zero with more or less marked positive and negative deviations due to experimental errors. A complication arises in the magnesium system where there are sometimes two well-defined linear phases after the explosion proper (see Fig. 6). The time intercept of the first of these is $t_{s,1}$ and of the second $t_{s,2}$ with velocities $V_{m,1}$ and $V_{m,2}$ respectively. Since the former lasts for at least 40 sec. and often much longer, it is clearly quite distinct from the much earlier explosive phase described by Weber & Hasselbach (1954) and by Tonomura & Kitagawa (1960) and for this reason will be treated separately below.

The mean values for $t_{s,1}$, $t_{s,2}$, $V_{m,1}$ and $V_{m,2}$ for the magnesium system are given in Table 3, and the values of t_s and V_m for the calcium system in Table 4. It is seen that t_s is generally very small indeed for the latter system, except in two anomalous cases at 0°, I 0·04 and I 0·25, where the high values are possibly due to swelling of the fibrils under these conditions. In all other cases the standard error is so large in comparison with the means that t_s frequently becomes positive instead of negative, and in two cases at 18° even the mean is positive. This is exactly what would be expected if t_s were really zero and the deviations were due to experimental error. We may conclude that no explosive phase exists in the calcium system when due allowance is made for inhibition by the products.

The magnesium system is rather more complex; Table 3 shows that t_s is generally negative, although often very small, and with a high standard error. At 0° swelling effects may again become a complicating factor, but even here there is generally a well-defined stationary phase lasting 2–4 min., of which the time intercept, $t_{s,1}$, is small in comparison with the very low velocities of hydrolysis. The essential difference between the two systems lies, however, in the much more pronounced shortening of the fibrils which occurs at the higher temperatures in the magnesium system as soon as ATP is added (cf. Marsh, 1952; Bendall, 1958). This shortening, which probably occurs during the first 5 sec. of the reaction and which often amounts to 70 % or more of the original length of the fibrils, would be expected to lower the velocity of hydrolysis, because in the supercontracted state many of the active enzyme sites would be masked by other inactive groups (see Hanson & Huxley, 1955). In spite of this, Weber & Hasselbach (1954) claimed in their original work that fibrils pretreated with ATP, i.e. pre-shortened, still showed an explosive phase on readdition of substrate. The present experiments do not confirm this view, but indicate exactly the contrary, as we see from Table 5, where the fibrils were pretreated with ATP + $MgCl_2$ under the appropriate ionic conditions at 18·5° and then washed free of substrate and products before the actual measurement of the rate of hydrolysis. The centrifuged volume of such fibrils was approximately half that of untreated fibrils, showing that considerable synaeresis had occurred. In every case, in Table 5, $t_{s,1}$ is reduced or abolished by the

pretreatment and $V_{m,1}$ is also generally lower than the control values. $V_{m,2}$, however, is not so much affected, and, at 35° and I 0·15, is actually higher than the control value and nearly equal to $V_{m,1}$. The effect of these changes is, of course, to reduce $t_{s,2}$ markedly in every case. We may conclude from these observations that the so-called explosive phase mainly represents the decreasing velocity of hydrolysis as the fibrils shorten and supercontract. Because this happens so quickly it becomes impossible to estimate accurately the true initial velocity of hydrolysis, V_o, although it is doubtful whether this could ever exceed $2V_{m,1}$. Fibrils pretreated with ATP and $CaCl_2$ and subsequently activated with $CaCl_2$ show no change in V_m, whereas pretreatment with ATP and $MgCl_2$ reduces V_m from ~14 to 13 units/sec. when hydrolysis is subsequently measured in the presence of Ca^{2+} ions at I 0·15 and 35°.

Energy of activation of hydrolysis

It is of some interest to calculate the apparent energy of activation (E_A) of the hydrolysis of ATP by the fibrils in the magnesium and calcium systems, because so far there are very few reliable data in the literature upon it. This can be done by means of the differential form of the Hood–Arrhenius equation

$$E_A = -R\frac{\mathrm{d}(\ln V_m)}{\mathrm{d}(1/T)}, \qquad (10)$$

where V_m is the steady velocity of hydrolysis and E_A is given in kcal./mole. The results based on the average $V_{m,1}$ or V_m values given in Tables 3 and 4 respectively are plotted as log V_m against $1/T$ in Fig. 8, and the E_A values calculated for the two temperature ranges 0–18·5° and 18·5–35° are summarized in Table 6. The average E_A values are always much higher in the magnesium than in the calcium system and moreover behave in an anomalous manner, falling in every case by ~6 kcal. as the temperature is raised from one range to the other. The values for the calcium system on the other hand are of the order to be expected of a straightforward chemical or enzyme-catalysed reaction (Moelwyn-Hughes, 1947; Dixon & Webb, 1958), that is ~11 kcal./mole, although they are also anomalous at low I, whereas at the higher they actually increase with increasing temperature (cf. Fig. 8 and Table 6). This second type of anomaly, an increase of E_A with temperature, occurs rarely, even in enzyme reactions (Dixon & Webb, 1958). The case of the fumarase reaction at high pH, reported by Massey (1953), was the only example of it known when Dixon & Webb were writing in 1958, and in that case it was probably due to a change of state of the enzyme.

It would be of great theoretical interest to be able to calculate the heat of combination of substrate or products with the enzyme, but this cannot be done from the data because only relative constants are available, i.e. K_s/K_{ADP}, K_s/K_{P_i} and the combined constant K'. This stresses the need for an accurate method for the measurement of K_s or K_m in this system without which neither the free energy nor the entropy of the substrate–enzyme or product–enzyme equilibria can be calculated. Some preliminary experiments have shown that K_s in the magnesium system at 35°

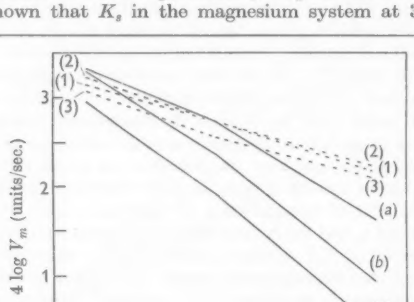

Fig. 8. Plot of log V_m against $1/T$ for the magnesium and calcium systems, based on the average $V_{m,1}$ and V_m values given in Tables 3 and 4 respectively. - - -, Calcium system: curves (1), (2) and (3) at I 0·042, 0·15 and 0·25 respectively. ——, Magnesium system: curves (*a*), (*b*) and (*c*) at I 0·042, 0·15 and 0·25 respectively.

Table 6. *Apparent energy of activation (E_A) of the splitting process, calculated from $V_{m,1}$ and V_m in Tables 3 and 4 respectively*

E_A is calculated as an average value over the stated range of temperature.

		E_A (cal./mole)		
Temp. range	Ion	I 0·042	I 0·150	I 0·250
0·6–18·5°	Mg	21400	28600	30100
18·5–35·0		14800	24200	26400
1·0–18·5	Ca	11400	10600	9400
18·5–35·0		10700	12100	13300

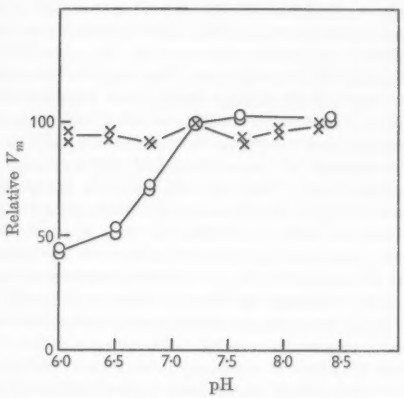

Fig. 9. Effect of pH on the steady velocity of hydrolysis, V_m, in the calcium and magnesium systems at 18·5°, I 0·15 and ATP 4 mM. Relative V_m values are given as % of V_m at pH 7·20. There were two experiments at each pH. ×, ATP + 8 mM-$CaCl_2$; ○, ATP + 4 mM-$MgCl_2$. Absolute V_m at pH 7·2 for calcium system was $5{\cdot}30 \times 10^{-2}$ unit/sec., and for magnesium system $2{\cdot}30 \times 10^{-2}$ unit/sec.

and I 0·04 is 0·089 mM (S.E. ± 0·007, n 15), but values at other temperatures have not yet been obtained.

Effect of pH

The effect of pH has been studied only under the rather special conditions of I 0·15 and 18·5°. Fig. 9 shows that the optimum range of pH for the magnesium system extends from pH 8·0 down to about 7·00, below which the relative velocity falls to a value of ~ 50 % of the maximal at pH 6·5 and is not much further reduced even at pH 6·0. In the calcium system, on the other hand, changes of pH within this range seem to have little or no effect on the rate of hydrolysis. Although it is unwise to generalize from only one value of I and temperature, it is probable, at least, that a pH of 7·2 is optimum for both systems. A wider range of values is clearly required, however, to establish whether the pH effect is due to dissociation of groups on the enzyme or on the substrate, which is an $(ATP–Mg)^{2-}$ complex in the magnesium system, but probably the free ion, ATP^{4-}, in the calcium system (Weber, 1959).

DISCUSSION

Most of the studies of the adenosine-triphosphatase activity of rabbit-muscle fibrils or of their constituent proteins, myosin and actomyosin, have been carried out at temperatures between 0° and 25° and often at very high or very low I (cf. Perry & Grey, 1956; Hasselbach, 1956; Morales & Botts, 1956; Blum, 1955). Such conditions diverge more or less widely from the physiological and this divergence has been further exacerbated in some cases by studying the reaction at pH 6·5 (e.g. Weber, 1959), which is certainly below the optimum pH for the magnesium-activated reaction (Fig. 8) and is also below the pH which can actually be measured in surviving muscle (Bate-Smith & Bendall, 1949; Bendall & Davey, 1957; Caldwell, 1958). From evidence that has accumulated over many years, it seems likely that the actual conditions in resting rabbit muscle, i.e. without an appreciable content of lactic acid, are a pH of 7·10–7·30, an I of 0·15–0·20 (Dubuisson, 1950), a concentration of Mg^{2+} ions of ~ 10 mM and of Ca^{2+} ions of ~ 2 mM, a concentration of ATP of ~ 10 mM and of ADP of ~ 1 mM, and a temperature of ~ 38° (cf. Hasselbach, 1957; Bendall & Davey, 1957). Of the soluble constituents, the ADP is bound to actin and most of the Ca^{2+} ions to actomyosin or the sarcoplasmic proteins, whereas most of the Mg^{2+} ions and ATP are not so bound, but are present as an $(ATP–Mg)^{2-}$ complex (Smith & Alberty, 1956; Hasselbach, 1957). It has been attempted here to approach these conditions as closely as possible or at least to cover a sufficient range to include them, because otherwise it is impossible to relate the results to the real state of affairs in living, contracting mammalian muscle.

Other points of great technical importance which have often been overlooked in previous studies are first the necessity of preparing adequate blanks and secondly of measuring the rate of hydrolysis from the earliest seconds of the reaction onwards. Lack of attention to the first point can lead to quite false deductions about the extent of the explosive phase, and to the second to an erroneous and incomplete interpretation of the effects of changing the environment, particularly in cases where the products inhibit the forward reaction. In the present study it became very noticeable, as the work proceeded and insight was gained in overcoming these difficulties, that the magnitude and duration of the explosive phase progressively decreased, until its apparent time intercept (t_s) was reduced from values of the order of tens of seconds to the very low values shown in Table 3 for the magnesium system and to the vanishingly small values in Table 4 for the calcium system. Coupled with this marked decrease was the observation that the explosion in the magnesium system could be virtually abolished by pretreatment of the fibrils with ATP; in other words, the falling velocity, characteristic of this phase of the reaction, appears to be entirely an effect of the concomitant supercontraction of the fibrils. It is significant that this effect is unique to the magnesium system because it is precisely here that the hydrolysis process

probably involves sites on both the actin and myosin filaments of the fibril, whereas in the calcium system it involves only sites on the myosin filaments (Bárány & Bárány, 1960; Nagai, Makinose & Hasselbach, 1960). If such is the case, it would be expected that supercontraction in the former system would have a considerable disorientating effect on the two enzyme sites involved, leading to a fall in the hydrolysis rate, but would have little or no effect in the latter, where only one type of site was involved. The view of Bárány & Bárány that only myosin sites are involved in hydrolysis in the calcium system is, incidentally, confirmed by the present results, because the relative inhibition constant, K_s/K_{ADP}, for this system at I 0·15 and 18·5° is ~0·70, which is close to the value of 0·90 obtained by Green & Mommaerts (1954) for highly purified myosin under nearly identical conditions. By contrast, the magnesium system shows no inhibition by the products under these conditions.

The extent of the disorientation during supercontraction in the magnesium system can best be envisaged from the sliding-filament model of Hanson & Huxley (1955), from which it is clear that even at ~30% shortening the I-bands of the sarcomeres have already vanished, the actin filaments of each half sarcomere have met in the middle of the A-band and are piling up into a contraction band, and the Z-disks are just in contact with the edges of the A-band. Shortening beyond ~70% of the initial length leads to further folding and buckling of the actin filaments and to buckling of the myosin filaments of the A-band. It is quite clear that these changes will rapidly and drastically reduce the number of opposing active sites on the two sets of filaments with a consequent fall in the velocity of hydrolysis of ATP from V_o to $V_{m,1}$ in the early 'explosive' phase. It is quite possible that further buckling of the filaments will occur in the supercontracted fibrils with time, which would account for the subsequent characteristic fall of velocity from $V_{m,1}$ to $V_{m,2}$ at ~40 sec. from the start (cf. Fig. 6). These changes in velocity are more clearly defined with frog-sartorious fibrils than rabbit fibrils, because V_o takes longer to decay to a plateau, $V_{m,1}$, and this plateau lasts only a short time before decaying at a slower rate to $V_{m,2}$. The two decay curves, $V_o \rightarrow V_{m,1}$ and $V_{m,1} \rightarrow V_{m,2}$, are of typical exponential form, $dV/dt = k(V_1 - V_2)e^{-kt}$. This, in itself, indicates that the change in velocity is unlikely to be due to a faulty product-inhibition equation, because there are no terms, even in the equation for non-competitive inhibition, which could account for the time course of the changes.

The general question of inhibition by the products at low I seems to have been studied only by Green & Mommaerts (1954), using myosin adenosine triphosphatase, and no data have hitherto been available on actomyosin or on the myofibrillar adenosine triphosphatase. The results presented here are unfortunately incomplete because they include no measure of K_s, so that only relative constants can be given, and these are subject to error because of the differential method used to calculate them. They give, however, a fairly clear idea of the general course of the reaction under the different conditions. Tables 1 and 2 show that K_s/K_{ADP} and the combined constant, K', generally tend to rise and K_s/K_{P_i} to fall with increasing temperature, whereas all three constants fall with I. The latter phenomenon is very striking in the magnesium system, where K' falls to zero at the two higher I values at temperatures of 18·5° or below, but is measurable in all cases at the lowest I. An exception to these general rules occurs in the calcium system at the highest I, where K' falls, instead of rising, as the temperature is increased, and this reversal is accompanied by an anomalous rise in the energy of activation of hydrolysis with temperature (cf. Table 6). By analogy with other systems this strongly suggests a change of state of the enzyme as the temperature is increased (Dixon & Webb, 1958), which in this case may be a change in the myosin filaments from the gel to the sol state or vice versa.

The energy of activation of the hydrolysis process has not been studied systematically in previous work, although Hasselbach (1952) and Perry & Grey (1956) have noted that the magnesium system in the temperature range 0–25° is much more strongly activated by increasing temperature than is the calcium system. The question seems worthy of more detailed investigation than this, however, because it bears not only on the nature of the process, but also on its role in the concentration cycle *in vivo*. As we have shown, the effect of Mg^{2+} ions always dominates that of Ca^{2+} ions in determining the initial velocity and the precise course of hydrolysis when both ions are present together *in vitro*, and this dominance is likely to be emphasized *in vivo* by the higher total concentration and the much higher 'free' concentration of Mg^{2+} ions than of Ca^{2+} ions, the latter being mainly bound to protein (Hasselbach, 1957). It would seem obvious, therefore, that it is the magnesium-activated process which is relevant in interpreting the contractile process *in vivo* and not the calcium-activated one, which has nevertheless been so persistently studied from this point of view (Green & Mommaerts, 1954; Mommaerts & Green, 1954; Morales, Botts, Blum & Hill, 1955). Table 6 shows that it is precisely the magnesium system that shows a high apparent energy of activation (E_A), particularly at physiological I values of ~0·15,

where it lies in the range 22–28 kcal./mole, and the calcium system which shows a normal E_A of ~11 kcal./mole, characteristic of many straightforward enzymic reactions (Dixon & Webb, 1958). Further, the magnesium system is anomalous in showing a very high average value for $\Delta E_A/\Delta T$ of more than -200 cal./mole/degree, which is at least three times the value for most of the so-called anomalous chemical and enzymic reactions (Moelwyn-Hughes, 1947; Dixon & Webb, 1958). Anomalies of this type are generally due to side reactions concurrent with the main one, or in enzyme-catalysed reactions to many reaction steps. In the present case, it has been suggested that the reaction takes place between active sites on the actin and myosin filaments of the fibril (Weber, 1958; Bárány & Bárány, 1960), and, if this is so, we can easily imagine that a number of steps would be necessary. Starting, as we must, in the system *in vitro*, with the fibrils in rigor and the overlapping actin (A) and myosin (M) filaments bonded together in a complex, A–M, we can formulate the initial steps as follows:

$$\text{A–M} + \text{ATP} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \text{A–ATP–M}, \qquad (a)$$

$$\text{A–ATP–M} \underset{K_{b,2}}{\overset{K_{b,1}}{\rightleftarrows}} \text{A} + \text{ATP–M}, \qquad (b)$$

$$\text{A–ATP–M} \underset{K_{c,2}}{\overset{K_{c,1}}{\rightleftarrows}} \text{A–ATP*–M}, \qquad (c)$$

$$\text{A–ATP*–M} \underset{K_4}{\overset{K_3}{\rightleftarrows}} \text{A} \begin{matrix} \overset{\text{ADP*}}{\diagdown\!\diagup} \\ \underset{\text{P}_i}{\diagup\!\diagdown} \end{matrix} \text{M}, \qquad (d)$$

$$\begin{matrix} \text{ADP*} \\ \diagup\!\diagdown \\ \text{A} \qquad \text{M} \\ \diagdown\!\diagup \\ \text{P}_i \end{matrix} \underset{K_6,\ K_8}{\overset{K_5,\ K_7}{\rightleftarrows}} \text{A–M} + \text{products}, \qquad (e)$$

where we have kept to the numbering of the constants in the simple formulation given in the section on theoretical analysis of the curves, and where the asterisks indicate activated complexes. We must also envisage, of course, a contraction step somewhere in the series, possibly at step (*d*) or between steps (*c*) and (*d*), because we know that supercontraction takes place at 18·5° and above. Similarly, step (*b*), an alternative type of dissociation of the enzyme–substrate complex, is obligatory because swelling of the fibrils, that is separation of the actin and myosin filaments by water of hydration, occurs under some conditions, particularly at the higher I values at 0° (unpublished work) and it also occurs whenever the forward reaction of step (*d*) or of earlier steps is artificially inhibited (Marsh, 1952; Bendall, 1958). It seems to be represented in the behaviour of intact fibre models by the process of relaxation, which also occurs whenever the early steps are inhibited (Weber, 1958). Indeed, quite apart from the contribution of the many other steps which could be formulated, this dissociation of actomyosin is probably the one which mainly determines the high E_A of the overall process. As will be shown, it occurs to a greater or lesser degree at all three values of I even in the absence of ATP, when it has a negative heat of dissociation in every case, that is the dissociation of A–M decreases with temperature. Taking all these considerations into account, it is not surprising that the E_A of the magnesium-activated process is extremely high and, moreover, rises as I increases, that is as the actomyosin dissociation increases. The calcium-activated process is, by contrast, very much simpler, first because little or no contraction occurs concomitantly with it and secondly because it behaves as if it involved only myosin filaments. In line with this view, we have shown that it is disturbed only slightly by previous supercontraction of the fibrils and further that its E_A is in the normal range, despite the considerable swelling accompanied by actual extraction of actomyosin from the fibrils, which can occur, particularly at 0° and at 18·5° and I 0·250. We can formulate its first steps as

$$\text{AM} + \text{ATP} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \text{AM–ATP} \longrightarrow \text{AM–ATP*}, \qquad (a')$$

$$\text{AM–ATP*} \underset{K_4}{\overset{K_3}{\rightleftarrows}} \text{AM} \begin{matrix} \overset{\text{ADP*}}{\diagdown} \\ \underset{\text{P}_i}{\diagup} \end{matrix}, \qquad (b')$$

and a dissociation step

$$\text{AM–ATP} \rightleftarrows \text{A–ATP–M} \rightleftarrows \text{A} + \text{ATP–M}. \qquad (c)$$

The dissociation reaction, however, has only a minor disturbing effect on V_m here in contrast with its very large effect in the magnesium system. Nevertheless, it may be this reaction which leads to the second and unique type of anomaly found in this system at I 0·150 and I 0·250, where E_A rises with temperature (cf. Table 6). We can easily imagine that the partial change of state of the myosin from gel to sol, which occurs respectively at 0° and 18·5° at these values of I, would increase the rate of hydrolysis out of proportion to the rate at 35°, where the myosin filaments probably remain intact during the course of the reaction.

We may conclude from the present results how important it is to study the reaction under conditions as nearly physiological as possible. To give an example of the confusion that may otherwise result, we have only to consider the steady velocities of hydrolysis (V_m) at physiological I (0·150) in the two systems. At 18·5°, for instance, the

ratio of V_m for the calcium system to V_m for the magnesium system is > 2, but at 35° it is only 0·8, because of the great difference in the energies of activation (cf. Tables 3, 4 and 6). Indeed, the true initial velocity of uncontracted fibrils in the magnesium system at 35° is probably at least $1\frac{1}{2}$ times the V_m for the supercontracted fibrils in Table 3, so that under living conditions this system hydrolyses ATP nearly twice as fast as the calcium system, which is of course unaffected by the process of supercontraction. Allowing for a temperature of 38°, we may calculate the maximum velocity of hydrolysis in the living animal to be $\sim 0{\cdot}44$ μmole of ATP/sec./mg. of fibrillar N, or $\sim 5 \times 10^{-4}$ mole/g. of fresh muscle/min. This is of the same order as the value of 10×10^{-4} calculated by Mommaerts (1950) from data for oxygen consumption, which necessarily involves a great number of assumptions. It is unfortunate, indeed, that we possess no reliable values for the heat production in a mammalian twitch with which to compare these rates of hydrolysis, whereas in the frog we have Hill's very accurate data for heat production (Hill, 1949) but little or no data for adenosine-triphosphatase activity. It is only when such data become available that it will be possible to test critically the various hypotheses concerning the role of ATP in contraction.

SUMMARY

1. The time course of the hydrolysis of adenosine triphosphate by rabbit myofibrils has been studied at temperatures of 0°, 18·5° and 35° and at ionic strengths (I) between 0·04 and 0·25, with Ca^{2+} ions, Mg^{2+} ions or a mixture of both as activators.

2. It has been found that the products inhibit the reaction, the inhibition being more pronounced in the calcium than in the magnesium system. The combined relative inhibition constant,

$$K_s/K_{P_i} + K_s/K_{ADP},$$

varies in the former system from $\sim 1{\cdot}0$ to 0° to about 2·2 at 35° for I 0·042, and from $\sim 0{\cdot}8$ to $\sim 0{\cdot}7$ for the same temperature range at I 0·250. In the magnesium system, no inhibition by products occurs at $I \geqslant 0{\cdot}150$ at the two lower temperatures, but the inhibition constant at $I \leqslant 0{\cdot}150$ reaches values of $\sim 1{\cdot}00$ at 35°.

3. When allowance is made for inhibition by the products in the calcium system, the so-called early explosive phase of the reaction is either absent or restricted to < 2 sec.; in the magnesium system a short explosive phase is always detectable, lasting for < 5 sec. at 18° or above, but is either absent or much reduced in fibrils supercontracted by pretreatment with adenosine triphosphate. It is suggested that the declining velocity during the explosive phase in this system is entirely due to reduction in the number of active enzyme sites during the process of supercontraction.

4. The progress curves of the calcium system and the product inhibition constants characteristic of it can be entirely converted into those characteristic of the magnesium system by addition of Mg^{2+} ions to one-seventh of the concentration of the Ca^{2+} ions. The explosive phase of the magnesium system and the characteristic supercontraction appear concomitantly. It is deduced that the Mg^{2+} ion is always the dominant ion when both ions are added together. It follows that it is the characteristics of the magnesium system which must be taken into account *in vivo*, where most of the Mg^{2+} ions are free and most of the Ca^{2+} ions are bound to protein.

5. The energy of activation of the hydrolysis process is ~11 kcal./mole in the calcium system, whereas it always exceeds 15 kcal./mole in the magnesium system, and *in vivo* is > 20 kcal./mole. It is suggested that these large differences are due to participation of sites on both the actin and the myosin filaments in the magnesium-activated process, but of only myosin sites in the calcium-activated.

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The Isolation of a New Lipid, Triphosphoinositide, and Monophosphoinositide from Ox Brain

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The classic work of Folch (1942) showed that inositol was a constituent of brain phospholipids and an inositol phosphatide fraction was isolated which was rich in these inositol lipids. This inositol phosphatide was subsequently subfractionated with solvents, and an inositol-containing phospholipid was isolated which was designated diphosphoinositide because it produced inositol diphosphate as a major product on acid hydrolysis (Folch, 1949). Also, a trypsin-resistant protein fraction was isolated from brain tissue. This fraction contained a phosphoinositide bound to it by a salt-like linkage so that it was not extracted with lipid solvents unless these were acidified (Folch, 1952; Lebaron & Folch, 1956).

The starting point of the present investigation was an attempt to prepare pure diphosphoinositide as a substrate for metabolic experiments. It was soon realized that the brain phosphoinositide fraction was heterogeneous and from it were isolated two other inositol-containing phospholipids, monophosphoinositide and a new lipid designated triphosphoinositide because it contains inositol and phosphate in the molar ratio 1 : 3. The fractionation of these inositol phospholipids was followed by examining ionophoretically the products they formed after deacylation by mild treatment with alkali (Dawson, 1960). This showed that only the monophosphoinositide was extracted from brain tissue with neutral lipid solvents such as chloroform–methanol unless the tissue was pretreated with acetone. This has led to a convenient method for the isolation of triphosphoinositide by removing it with acidified lipid solvents from brain tissue previously extracted with neutral solvents. The protein–triphosphoinositide complex obtained is then disrupted to liberate the free lipid.

This paper is concerned with the isolation of triphosphoinositide from brain tissue and describes some properties of the new lipid. It also reports the isolation and analysis of monophosphoinositide from the same tissue. A preliminary account of this work has been given (Dittmer & Dawson, 1960). Confirmation of the existence of triphosphoinositide in brain has also been recently obtained independently by Grado & Ballou (1960, 1961), who isolated inositol triphosphate from acid hydrolysates of purified diphosphoinositide preparations.

EXPERIMENTAL

Materials

Ox brain was obtained from the animal as soon as possible after death and transported to the laboratory packed in ice. No attempt was made to separate white and grey matter for the fractionation. Guinea-pig brains were removed from the animals a few minutes after killing with chloroform and accumulated in solid CO_2.

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Isolation of monophosphoinositide and triphosphoinositide from acetone-pretreated brain tissue

The brain tissue was stripped of adhering blood clots and then treated with acetone and ethanol and extracted with light petroleum according to Folch (1942, 1949). The ethanol-insoluble phosphoinositide fraction was subjected to multiple precipitation from chloroform with methanol to obtain the 'diphosphoinositide' fraction (Folch, 1949). The soluble material from all these methanol precipitations was combined, dried *in vacuo* and dissolved in chloroform. Monophosphoinositide was isolated from this chloroform solution by chromatography on silicic acid columns as described by Hanahan, Dittmer & Warashina (1957) or on alumina columns followed by silicic acid chromatography (Dawson, 1958).

The insoluble 'diphosphoinositide' fraction obtained by the multiple-precipitation procedure described above was dissolved in chloroform–methanol (4:1, v/v) and transferred to a column of Amberlite IR-120 (H+) resin which had been previously washed with chloroform–methanol (4:1, v/v). Approximately 4 ml. of resin was used/mg. of organic P in the preparation. The phospholipid was washed through the column with chloroform–methanol (4:1, v/v) by using 4 ml./ml. of resin. This treatment effectively removes cations and also 90–95% of the inorganic P which is usually present to the extent of 50% of the total P. This may be due to a combination of the phosphate with bivalent cations such as Ca^{2+} or Mg^{2+} adsorbed on the sulphonate groups of the resin.

The eluent from the ion-exchange resin was dried under vacuum and a countercurrent distribution carried out in a solvent system consisting of chloroform, ethanol, ether and water in the proportions of 3:2:5:2 by vol. Four transfers were usually sufficient to effect good separation of an inositol fraction in the upper layer although a little triphosphoinositide remained in the chloroform or bottom layer of the system. Emulsions formed in the course of the fractionation were broken by centrifuging; accordingly, it was found most convenient to carry out the fractionation in centrifuge tubes. Transfer of the top fractions was facilitated by drawing them off with a siphon bottle connected to a water aspirator. The top layer from this fractionation was dried *in vacuo* (acetone was added to prevent foaming) and the triphosphoinositide dissolved in moist chloroform.

Application of the same isolation procedure to fraction I described by Folch (1942) rather than to the purified 'diphosphoinositide' (8 ml. of resin/mg. of organic P) produced a preparation which contained in addition to triphosphoinositide a nitrogenous compound that has not yet been identified. This contaminant was, however, eliminated by the following procedure.

Fraction I was suspended in aqueous 50% (v/v) ethanol (2 ml. of 50% ethanol/mg. of lipid P) and saturated $Ba(OH)_2$ solution was added until the mixture was alkaline to phenolphthalein. This mixture was extracted twice with equal volumes of chloroform. The combined chloroform extracts were washed twice with water and the volume was reduced to approx. one-tenth. Methanol (4 vol.) was added and the precipitate produced after standing at −18° for 0·5 hr. was collected by centrifuging and was then dissolved in chloroform–methanol (4:1, v/v). The final purification of triphosphoinositide was carried out as described above starting with the passage of the chloroform–methanol solution through an Amberlite IR-120 column.

Preparation of triphosphoinositide from chloroform–methanol-extracted brain-tissue residue

Ox brain (200 g.) was freed from adhering blood clots and homogenized in a Waring Blendor in 2 l. of chloroform–methanol (1:1, v/v). After 10 min. the suspension was centrifuged, the insoluble matter was washed with 1 l. of chloroform–methanol (2:1, v/v) and again recovered by centrifuging. The residue was extracted three times with 400 ml. volumes of chloroform–methanol (2:1, v/v) each containing 1 ml. of conc. HCl. The extraction was carried out at 37° with stirring and for 20 min. periods. The triphosphoinositide was only slowly extracted under these conditions and hardly at all at room temperature. The combined acidified chloroform–methanol extracts containing a good deal of haem pigments were shaken with 0·2 vol. of 0·9% NaCl. After centrifuging, the upper aqueous phase was sucked away and discarded, the layer of interfacial white material penetrated and the lower chloroform phase removed by suction and collected. The interfacial material containing most of the triphosphoinositide was saved. The lower chloroform layer was shaken with 0·2 vol. of 0·9% NaCl, and, after centrifuging, a small amount of interfacial material was collected. The lower chloroform phase containing the haem pigments and traces of lipids not removed in the original extraction was discarded.

The combined interfacial material fractions were shaken with 400 ml. of chloroform–methanol (2:1, v/v) and 80 ml. of 0·9% NaCl, and, after centrifuging, the interfacial material was collected. To the interfacial material was added 80 ml. of acetone, the mixture was heated to boiling for 2 min. and then dried *in vacuo*. The residue was treated with 80 ml. of ethanol, which was evaporated off on a boiling-water bath and finally *in vacuo*. The residue was extracted twice with 200 ml. of chloroform–methanol (2:1, v/v) containing 0·1 ml. of conc. HCl. The mixture was initially homogenized in a blender to break up any lumps and the extraction carried out for 5 min. at 37°. The extracts were filtered through a pad of glass wool (filter paper often resulted in low yields and its use was avoided throughout the fractionation). The combined extracts were vigorously shaken with 0·2 vol. of N-HCl and, after centrifuging, the lower layer containing the crude triphosphoinositide was collected. To remove protein the chloroform layer was mixed with 0·5 vol. of methanol and shaken with 0·2 vol. of N-HCl. The lower layer was collected, any interfacial material was rejected, and 0·5 vol. of methanol added. The solution was shaken with 0·2 vol. of water and the lower layer collected after centrifuging. It contained crude triphosphoinositide corresponding to 3·17–9 mg. of P in various preparations.

The crude triphosphoinositide was now purified via its sodium or ammonium salt. The lower chloroform layer was dried *in vacuo* below 35° and dissolved in 3 ml. of chloroform. In some preparations a clear solution was not obtained. This could often be remedied by adding an equal volume of methanol, drying *in vacuo* and redissolving in the same amount of chloroform. Any solid matter remaining was removed by centrifuging. To the clear brownish solution 20 ml. of methanol was added. After 2 hr. at −15° any precipitate was centrifuged off and rejected. It usually

contained only a small percentage of the total P and analysis showed it to be largely crude triphosphoinositide. To the supernatant, cooled in an ice bath, was slowly added methanolic NaOH (0·1N) until a pH of 6·5–7 was reached and precipitation of the sodium salt was complete. Care had to be taken to avoid excess of alkali or deacylation resulted. In this respect preparation of the ammonium salt is preferable and this was simply done by passing dry NH_3 gas into the supernatant until a slight excess (pH 8) was present. After several hours at −10° the sodium or ammonium triphosphoinositide was centrifuged off and a small extra amount could be obtained by working up the mother liquors. The compound was then reprecipitated from chloroform solution with methanol (or acetone), centrifuged off and dried or stored in chloroform solution.

Analyses

Glycerol. This was assayed according to the procedure of Hanahan & Olley (1958). The lipid was hydrolysed with 2N-HCl in a sealed tube for 48–72 hr. at 105° to prevent loss of glycerol by volatilization or decomposition. Assays were done in triplicate and a standard curve was run with each set of determinations.

Inositol. This was determined microbiologically with *Kloeckera brevis*, by the method of Campling & Nixon (1954), as described by Dawson & Freinkel (1961). Lipid samples were hydrolysed for 30–48 hr. in 5N-HCl at 105°, the acid was removed by evaporation to dryness *in vacuo*, and the residue dissolved in water and adjusted to pH 5 with NaOH for assay.

Phosphorus. This was estimated by the method of Fiske & Subbarow (1925) after digestion with $HClO_4$.

Acyl ester groups. These were determined by the method of Stern & Shapiro (1953) scaled down so that the colour was developed in 1·0 ml. total final volume and read in a microcell (1 cm. path) of the Unicam SP. 500 spectrophotometer. A standard curve with 0–0·5 μ-equiv. of triacetin was determined with each set of analyses. Pure ovolecithin gave the theoretical P:acyl ester ratio.

Fatty acid. The lipid residue was hydrolysed for 1 hr. at 100° in 1 ml. of N-KOH and after cooling 1·5 ml. of N-HCl was added. The mixture was shaken with 3 ml. of light petroleum (b.p. 60–80°) and centrifuged, and a 2 ml. sample of the upper layer was carefully removed without touching the walls of the tube. To the solution of fatty acids was added 3·5 ml. of acetone and cresol red indicator. The acid was titrated with methanolic 0·005N-NaOH with a stream of N_2 passing through the solution. A standard curve was prepared with stearic acid.

Nitrogen. This was estimated by the procedure of Lang (1958).

Sodium. This was determined by using a flame photometer (EEL).

Acid and alkaline hydrolysis of lipids. The technique used and the separation of the hydrolysis products by paper chromatography and ionophoresis and column chromatography is described in the next paper (Dawson & Dittmer, 1961).

RESULTS

Examination of the isolated monophosphoinositide. On analysis the lipid contained 3·45% of P, 10·2% of glycerol, 20·6% of inositol and 0·08% of N. The acyl ester : P ratio was 2·06 : 1 and no reaction for plasmalogen was given. The values agree closely with the theoretical values for distearoylphosphatidylinositol, which contains 3·58% of P, 10·6% of glycerol, 20·8% of inositol, no nitrogen and an acyl ester : P ratio of 2 : 1.

The structure was more firmly established by an analysis of the products obtained after mild hydrolysis with alkali and acid. With the former (5 min. at 37° in 0·25N-NaOH) the major phosphorus-containing product was glycerylphosphorylinositol. Of the total phosphorus of the hydrolysate 85% was present as glycerylphosphorylinositol, an additional 4% was found as inositol monophosphate and 0·7 and 4% was present as glycerophosphate and cyclic glycerophosphate respectively. On longer hydrolysis (20 min.), less glycerylphosphorylinositol (62%) and correspondingly larger amounts of inositol monophosphate (21%) and cyclic glycerophosphate (18%) were detected. Mild acid hydrolysis of brain monophosphoinositide produced 77% of inositol monophosphate, 11% of glycerophosphate plus traces of inorganic phosphate. Additional glycerol was isolated as monoglyceride (42%) and diglyceride (28%). The hydrolysis products obtained by both acid and alkaline treatment suggest, and are consistent with, a structure for the monophosphoinositide of brain that corresponds to that found for liver monophosphoinositide, that is phosphatidylinositol (diacylglycerylphosphorylinositol).

Notes on the preparation of triphosphoinositide. During the development of procedures for the isolation of triphosphoinositide the fractionation was continually followed by acid and alkaline hydrolysis of the lipid fractions and the examination of the hydrolysis products by high-voltage ionophoresis. Brief acid hydrolysis (7 min. at 100° in 5N-HCl) was generally most useful and, as is described in the next paper (Dawson & Dittmer, 1961), yielded inositol triphosphate as the predominant phosphate ester.

The isolation of triphosphoinositide from the protein complex insoluble in neutral lipid solvents is probably the most useful for routine preparation. Over a dozen preparations have now been made in this way and a reasonable yield was obtained on each occasion. The other lipids, including a troublesome nitrogen-containing non-phospholipid contaminant of the preparation made from a diphosphoinositide fraction, are effectively removed in the initial lipid extraction with neutral solvents. In the preparation, protein can be a contaminant but the vigorous shaking with N-HCl of the chloroform–methanol solution of triphosphoinositide reduces this to very low limits. The most troublesome contaminant, however, appears to be neutral lipid material, consisting largely of diglyceride, which

Table 1. *Analysis of typical preparations of triphosphoinositide*

Species	Yield (mg./kg. of brain P)	Salt isolated	Acyl ester	Total fatty acid	Inositol	Glycerol
			(molecules/3 atoms of P)			
Guinea-pig	24·0	Na	—	—	1·1	1·13
Ox	—	Na	2·26	—	0·95	1·05
Ox	39·5	Na	2·02	1·97	0·98	0·93
Ox	38·7	Na	2·30	2·31	0·97	1·30
Ox	45·7	NH_4	2·10	2·11	1·13	1·07
Ox	—	NH_4	2·11	2·19	1·22	1·09
Ox	—	Free acid	1·92	—	1·05	0·94
Ox	30·7	Free acid	1·86	—	0·91	1·02

probably arises by breakdown of triphosphoinositide itself during the course of the preparation. This appears to form a complex with the triphosphoinositide so that under certain circumstances of precipitation of the sodium salt the acyl ester : P ratio is almost exactly 1 : 1. At first it was thought that this complex was another form of triphosphoinositide (triphosphoinositide B; Dittmer & Dawson, 1960), but certain evidence now suggests this is not so. Thus material rich in acyl ester and glycerol but containing no P can be eluted with chloroform when such preparations of triphosphoinositide are placed on a silicic acid column, and material rich in fatty acid and low in P is concentrated in the supernatant on precipitation with acetone. When the substance eluted from a silicic acid column with chloroform was subfractionated on a similar column with ether–light petroleum mixtures (Dawson & Dittmer, 1961), diglyceride was the predominant fraction emerging (acyl ester : glycerol ratio 2·01 : 1; no P or inositol).

Properties of isolated triphosphoinositide. The sodium salt of triphosphoinositide is a snow-white amorphous solid, which has not yet been crystallized. It is readily soluble in water, forming a clear solution with detergent-like properties. It is less readily soluble in wet chloroform, and is virtually insoluble in methanol, acetone and ether. The free acid is likewise soluble in water and chloroform and differs in being completely soluble in methanol and ethanol. The sodium salt showed no evidence of instability when stored in moist chloroform solution at −18°, whereas the free acid slowly decomposed, presumably by hydrolysis as the products were similar to those observed on acid hydrolysis of the lipid (inositol triphosphate is the main P-containing decomposition product).

Analysis of triphosphoinositide fractions. The sodium triphosphoinositide preparations obtained contained no detectable choline (Appleton, La Du, Levy, Steele & Brodie, 1953), sphingosine or chloroform-soluble long-chain bases (McKibbin & Taylor, 1949), ninhydrin-reacting material or galactose. The N : P atomic ratio varied between 0 and 0·08 : 1. Only traces of sulphur were present. The only polyol-reacting substances detected on chromatography of hydrolysates were inositol and glycerol. Table 1 shows the analysis of a number of typical preparations in terms of the molar ratio of phosphorus to the other components and indicates that the lipid contains phosphate, inositol, glycerol and fatty acid in the molar ratios of 3 : 1 : 1 : 2. The preparations isolated as the free acid (Table 1) were obtained by the fractionation of the lipids extracted from acetone- and ethanol-pretreated brain tissue with light petroleum (Folch, 1949). In 11 preparations the mean phosphate : inositol molar ratio was 3 : 1·02 ± 0·11 (S.D.). (The microbiological assay of inositol is subject to error of about ± 6 %.) All of the fatty acid is combined through a carboxylic acid ester linkage. Analysis of a preparation for Na showed the atomic ratio Na : P to be 3·83 : 3, which suggests that the lipid is probably isolated as its tetrasodium salt. If it is assumed that the basic unit of triphosphoinositide contains three phosphate groups, one each of inositol and glycerol, two fatty acids (stearic acid) and four Na atoms, then the dry-weight analysis agrees well with the calculated theoretical (e.g. Found: P, 8·6; inositol, 16·4; glycerol, 7·9. Required: P, 8·4; inositol, 16·2; glycerol, 8·3 %). Such dry-weight analyses indicate that all the components of the complex molecule have been accounted for, but owing to uncertainty of the mean molecular weight of the fatty acid components this evidence by itself is not entirely satisfactory.

DISCUSSION

The present results and those in the next paper (Dawson & Dittmer, 1961) clearly show that the phosphoinositide fraction of brain tissue is a complex fraction that probably contains at least three individual phospholipids. From it have been isolated monophosphoinositide and a new lipid which we have designated triphosphoinositide (Dittmer & Dawson, 1960). Additional evidence, which will be presented in the accompanying paper, suggests that a third lipid corresponding to the diphosphoinositide described by Folch (1949) may also be present. The

results therefore help to explain the recent observations of Hörhammer, Wagner & Hölzl (1960), who showed that 'diphosphoinositide' preparations split into three spots on chromatography on formaldehyde-treated paper.

There seems little doubt that the new lipid isolated, triphosphoinositide, is a chemical entity that is distinct from the diphosphoinositide described by Folch (1949). In 11 preparations the mean inositol : phosphate molar ratio was 3 : 1·02 and it is shown in the accompanying paper that inositol triphosphate is formed in 80 % yield on acid hydrolysis. In agreement with this, Grado & Ballou (1961) have described the isolation of inositol triphosphate from the acid hydrolysates of crude brain diphosphoinositide preparations.

Monophosphoinositide is a phospholipid that has been chemically characterized as a component of certain mammalian tissues such as liver (McKibbin, 1956; Dawson, 1958; Hanahan & Olley, 1958) and heart muscle (Faure & Morelec-Coulon, 1954, 1958; Gray & Macfarlane, 1958). Hörhammer *et al.* (1958, 1960) obtained paper-chromatographic evidence for the presence of monophosphoinositide in brain and also identified inositol monophosphate as a hydrolysis product. The present isolation and chemical characterization removes any remaining doubt as to the presence of this phospholipid in nervous tissue. Moreover, its acid and alkaline hydrolysis products (diglyceride, glycerylphosphorylinositol, inositol monophosphate, glycerophosphate) indicate that it also has the phosphatidylinositol structure (diacylglycerylphosphorylinositol), which is the same as the monophosphoinositide of other tissues.

Monophosphoinositide in brain tissue differs from the other phosphoinositides present in that it can be readily extracted by neutral chloroform–methanol solvent. Triphosphoinositide appears to be much more strongly bound to a protein-like component but it can be partially extracted either by pretreating the tissue with acetone or by acidifying the extracting solvent. Presumably acid breaks the linkage between the phospholipid and the protein by displacement whereas acetone may break it by denaturation of the protein. It seems likely that such a complex may be a component of the trypsin-resistant protein fraction containing combined inositol (phosphatido-peptide) studied by Folch (1952) and Lebaron & Folch (1956). Such a fraction, which is probably related to classical neurokeratin, was found to yield on hydrolysis fatty acid and a phosphate ester with a P : inositol ratio of 2·6 : 1.

It seems likely that triphosphoinositide may play an important metabolic role in the economy of nervous tissue since work with radioactive phosphate has demonstrated that the protein-bound P fraction (which is probably mainly this phospholipid) has a high exchange rate (e.g. Findlay, Strickland & Rossiter, 1954). No clue as to such a function is as yet available except perhaps the fact that the lipid is only present in significant concentrations in the nervous system.

SUMMARY

1. A new phospholipid designated triphosphoinositide has been isolated from ox and guinea-pig brain.

2. Analysis shows that this compound contains inositol, phosphate, glycerol and fatty acid in the molar ratios of 1 : 3 : 1 : 2. All of the fatty acid is acyl ester-linked. The lipid contains no nitrogen as an integral part of its molecule.

3. Evidence is presented that triphosphoinositide is tightly attached to brain protein. Such a complex is probably a constituent of the phosphatido-peptide fraction described by Folch (1952). Pretreatment of the brain tissue with acetone partially breaks the linkage in the complex, and triphosphoinositide then becomes a component of the 'diphosphoinositide' fraction of brain tissue.

4. Monophosphoinositide has been isolated from brain tissue, and chemically characterized. Its degradation products on acid and alkaline hydrolysis indicate that it has the phosphatidyl structure (diacylglycerylphosphorylinositol).

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Evidence for the Structure of Brain Triphosphoinositide from Hydrolytic Degradation Studies

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In the preceding paper (Dittmer & Dawson, 1961) we have described the isolation from brain tissue of a new lipid, triphosphoinositide, which contains inositol, phosphate, glycerol and fatty acid in the molar proportions 1 : 3 : 1 : 2. In this paper a study of the chemical hydrolysis products of triphosphoinositide is reported, from which it can be deduced that the phospholipid is a diacylglycerylphosphorylinositol diphosphate. Similar observations have also been made upon a 'diphosphoinositide' fraction obtained from ox brain by the method of Folch (1949), which suggests that it may contain, as well as triphosphoinositide, a lipid that corresponds to a true diphosphoinositide. Preliminary results in this study have already been reported (Dawson & Dittmer, 1960).

EXPERIMENTAL

Methods. Triphosphoinositide and other brain-phospholipid preparations were prepared as described by Dittmer & Dawson (1961). These preparations were usually stored in chloroform at −18°. Since under these conditions preparations of the free acid were found to undergo slow hydrolysis over a period of several weeks, the triphosphoinositide was usually freshly prepared for each experiment and used within a few days or alternatively it was stored as the sodium salt.

The term fraction I is used here to designate the phosphoinositide-rich precipitate obtained by the addition of ethanol to a chloroform solution of brain kephalin as described by Folch (1949). The term Folch 'diphosphoinositide' refers to the insoluble fraction obtained from fraction I after a sequence of precipitations from chloroform with methanol (Folch, 1949). Although no evidence was obtained for decomposition of these fractions when stored in chloroform at −18°, freshly prepared fractions were used for the hydrolysis studies described.

Analysis. The methods used for the estimation of glycerol, inositol, phosphorus and acyl ester were those described by Dittmer & Dawson (1961).

Acid hydrolysis of phospholipids. A sample of a chloroform solution of triphosphoinositide containing 250–500 μg. of P was dried *in vacuo* and hydrolysed for 5–180 min. with 2 ml. of N- or 5N-HCl at 105°. After cooling, chloroform (4 ml.) was added and, after shaking and centrifuging, the upper aqueous layer was withdrawn and dried *in vacuo*. The lower chloroform layer was reserved when necessary for examination of the lipid degradation products. In some experiments these were removed by extracting twice with ether instead of chloroform. Even after short hydrolysis periods the P was almost all in the aqueous layer.

Mild alkaline hydrolysis of phospholipids. Mild alkaline hydrolysis was carried out by the procedure described by Dawson (1960) or alternatively a sample of the lipid, containing approx. 500 μg. of lipid P, was suspended in 8–10 ml. of aqueous 80% (v/v) ethanol, 0·25 ml. of aqueous N-NaOH was added and the mixture incubated for 5–20 min. at 37°. Neutralization of the hydrolysate and subsequent preparation of the phosphate esters for chromatography was carried out as described by Dawson (1960). However, the use of Amberlite IRC-50 was preferable to neutralization with ethyl formate if the hydrolysate was to be examined directly by paper ionophoresis. Excess of sodium formate in hydrolysates that were spotted directly for paper ionophoresis resulted in poor resolution of the phosphate esters. No such effect was observed if a prior chromatographic run was made with a solvent system as in the two-dimensional systems described below.

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Separation of phosphate esters in hydrolysates. Phosphate esters were separated either by paper ionophoresis at pH 3·6, or two-dimensionally by a combination of paper chromatography in phenol–water–acetic acid–ethanol and ionophoresis as described by Dawson (1960). Details of the detection and estimation of phosphate ester spots are also given in that paper.

For isolation of the phosphate esters from single-dimensional ionograms, the hydrolysate was applied as a band and after ionophoresis for 90 min. at 40 v/cm. the volatile buffer was removed by drying. [Pyridine–acetic acid–water (1:10:89, by vol.), pH 3·6, was used as the buffer for acid hydrolysates; increasing the pyridine to 5 vol. gave a better separation for alkaline hydrolysates.] The paper was then dipped in a modified form of the Rorem (1959) reagent (0·025% quinine sulphate dihydrate in ethanol), washed twice with ethanol to remove excess of quinine sulphate, and the separated bands were then marked by their fluorescence in u.v. light. Each band was cut out and the quinine salt of the phosphate ester eluted chromatographically with water. In a few experiments the bands were located by spraying with an indicator (0·04% bromocresol green in ethanol) but this was not as sensitive as the quinine sulphate method.

Examination of the lipid acid-hydrolysis products. The lipids in the ether or chloroform extract of the acid hydrolysates were examined by two methods.

(1) The ether extract was washed with 4% Na_2CO_3 to remove free fatty acids. The ether extract was dried and the mono- and di-glycerides were separated by four transfers in a countercurrent distribution system of equal volumes of light petroleum (b.p. 40–60°) and aqueous 85% (v/v) ethanol (Olley & Lovern, 1954). The mono- and di-glyceride fractions were characterized by measurement of their acyl ester and glycerol content.

(2) The lipids were fractionated on a silicic acid column according to a simplified form of the method of Hirsch & Ahrens (1958). The column (10 cm. long × 2 cm. diam.) was treated with light petroleum (60–80° b.p.) and then successively eluted with 100 ml. of 8% (v/v) of ether in light petroleum, 100 ml. of 25% (v/v) of ether in light petroleum and finally with 50 ml. of ether.

RESULTS

Acid hydrolysis of triphosphoinositide. Fig. 1 (*a*) shows the separation of the phosphorus products obtained after hydrolysis of triphosphoinositide (prepared from chloroform–methanol-extracted brain residue) for 5 min. in 5N-HCl at 100°. After such hydrolysis all the free and combined fatty acids were removed by extraction with organic solvents so none of the phosphate esters examined contained fatty acids. The minor components, inositol monophosphate, glycerophosphate and inorganic phosphate, were identified by using markers. The two major components were identified by isolation and analysis. The large fast-running ionophoretic spot (*A*), identified as inositol triphosphate, contained no glycerol, and contained phosphate and inositol in the molar ratio 3 : 1·01 and, in a second isolation, 3 : 0·98. The spot *B* contained phosphate and inositol in the ratio 2 : 1·08 and no glycerol, which indicates that it is due to an inositol diphosphate. In five experiments the inositol triphosphate yields were 77, 81, 77, 70 and 77% of the water-soluble P after 5 min. hydrolysis; the inositol diphosphate amounted to about 15–20%, whereas the glycerophosphate was 4–5% and inorganic phosphate and inositol monophosphate were each less than 1%. Examination of the hydrolysate for P-free polyols (Dawson, 1958) indicated that it contained free glycerol and a little free inositol.

The ether-soluble lipid degradation products were applied to a silicic acid column and eluted with chloroform. The first fraction obtained contained 40% of the acyl ester in the original triphosphoinositide hydrolysed, the ratio of acyl ester : glycerol was 2·01 : 1·0, and no inositol or P was present. This indicated that at least 40% of

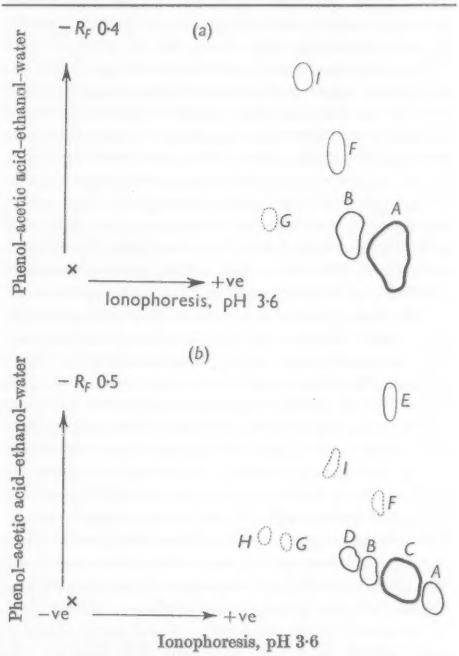

Fig. 1. Separation by paper chromatography and ionophoresis of (*a*) acid hydrolysis P-containing products and (*b*) alkaline hydrolysis P-containing products of triphosphoinositide. The thicknesses of the spots' outlines indicate relative intensities. Identity of spots: (*A*), inositol triphosphate; (*B*), inositol diphosphate; (*C*), glycerylphosphorylinositol diphosphate; (*D*), glycerylphosphorylinositol monophosphate (?); (*E*), cyclic glycerophosphate; (*F*), inorganic phosphate; (*G*), inositol monophosphate; (*H*), glycerylphosphorylinositol; (*I*), glycerophosphate.

the original acyl ester present in the lipid had been converted into diglyceride. In a second experiment the lipids, after being washed with Na_2CO_3 to remove free fatty acids, were run on a Hirsch & Ahrens (1958) column. Diglyceride was isolated in a yield of 30%; its acyl ester : glycerol ratio was 1·98 : 1·0.

When the triphosphoinositide prepared from a solvent-soluble 'diphosphoinositide' fraction (see Dittmer & Dawson, 1961) was examined a very similar pattern was found. Thus hydrolysis for 10 min. at 100° in N-HCl produced 75% of inositol triphosphate (inositol : P ratio, 1 : 3·1), 10% of inositol diphosphate (inositol : P ratio, 1 : 2·08), 6% of glycerophosphate and 6% of inorganic P and traces of inositol monophosphate. The distribution of glycerol in the hydrolysate was as follows, the lipids in this case being separated by the countercurrent distribution method: diglyceride (acyl ester : glycerol ratio, 2·07 : 1), 20%; monoglyceride (acyl ester : glycerol ratio, 0·96 : 1), 38%; glycerophosphate, 4%; free glycerol, 33%.

Experiments were performed to test how the phosphate-ester distribution changed on prolonging the acid hydrolysis. Table 1 shows that the inositol triphosphate disappeared, and at the same time there was an accumulation of inositol diphosphate, inositol monophosphate and a large amount of inorganic P. The glycerophosphate content of the hydrolysate did not change appreciably. If isolated inositol triphosphate was hydrolysed with 5N-HCl at 100° there was a slow accumulation of inositol diphosphate and inorganic phosphate.

Alkaline-hydrolysis products of triphosphoinositide. Mild alkaline hydrolysis of triphosphoinositide prepared from solvent-extracted brain tissue gave a relatively complex phosphate ester picture (Fig. 1*b*). A similar multiple breakdown has been observed to occur with phosphatidylinositol (Dawson, 1960). The products have been identified: (1) by the use of markers; (2) from the known behaviour of hydrolysis products of other lipids, e.g. phosphatidylinositol; (3) by isolation and analysis. The fastest-running spot on ionophoresis (*A*) had a mobility relative to inorganic phosphate which was identical with that of the main P-product of acid hydrolysis, i.e. inositol triphosphate. On analysis it contained no glycerol and a phosphorus : inositol ratio which was 3·0 : 1·04 and 3·0 : 0·98 in two separate isolations; thus it was confirmed that it was inositol triphosphate.

The major spot (*C*) contained phosphate, inositol and glycerol in the molar ratios 3 : 1·07 : 0·99 and 3·0 : 1·16 : 0·98 in two separate experiments. On acid hydrolysis (10 min. in 5N-HCl at 100°) *C* completely decomposed forming inositol triphosphate, glycerophosphoric acid and inositol diphosphate, the latter two compounds containing phosphorus in the concentration ratio of 1 : 2·05 respectively. The major phosphorus-containing product of mild alkaline hydrolysis is therefore probably a glyceryl-phosphorylinositol diphosphate. Under normal conditions of alkaline hydrolysis (Dawson, 1960) the compound was obtained in a yield of 44, 47, 46% in three experiments, but on using the modified method and avoiding acid conditions the yield was much higher (71, 78%).

Compound *B* (Fig. 1*b*) proved difficult to separate from *D*. On isolation the phosphorus : inositol ratio was 2·0 : 0·98 and 2·0 : 0·87 in two experiments but it also contained a little glycerol. From the relative mobilities and from evidence given later, spot *B* is probably due to inositol diphosphate and spot *D* to glycerylphosphorylinositol monophosphate. The latter may be formed from a true diphosphoinositide contaminating the preparation, as one would expect more inorganic phosphate to be present if it had been formed from triphosphoinositide. A little cyclic glycerophosphate is also present (*E*) and traces of inorganic phosphate (*F*), inositol monophosphate (*G*) and glycerylphosphorylinositol (*H*).

When triphosphoinositide preparations obtained from Folch's fraction I or purified 'diphosphoinositide' were examined by mild alkaline hydrolysis the phosphate esters obtained were very similar to those produced from the triphosphoinositide prepared from brain residue which had been extracted with chloroform–methanol. The major water-soluble phosphate ester on isolation had a molar ratio of glycerol : inositol : phosphate of 1·0 : 1·06 : 3·18, and 1·0 : 0·97 : 3·02 in two experiments. On acid hydrolysis (10 min. in 1N-HCl at 100°) of this compound, inositol triphosphate was formed as the major phosphorus-containing product as well as glycerophosphate and inositol diphosphate.

Examination of Folch's (1949) *fraction I and 'diphosphoinositide'.* Chromatography of mild alka-

Table 1. *Phosphorus-containing products in acid hydrolysate of triphosphoinositide*

Time of hydrolysis in 5N-HCl (min.)	Temp.	Percentage of total P as				
		Inositol triphosphate	Inositol diphosphate	Inositol monophosphate	Inorganic P	Glycerophosphate
10	100°	78	15	1	1	5
60	105	61	20	4	5	5
180	105	15	26	12	43	4

line and acid hydrolysates of Folch's (1949) fraction I of brain kephalin and purified 'diphosphoinositide' revealed a rather complex mixture of phosphorus-containing compounds. Both of these fractions produced on mild alkaline hydrolysis glycerophosphorylinositol diphosphate and glycerophosphorylserine which are undoubtedly derived from triphosphoinositide and phosphatidylserine respectively. Inositol triphosphate was also produced and was probably also derived from triphosphoinositide. Three products, glycerylphosphorylinositol, inositol monophosphate and glycerophosphate were found in the hydrolysates of fraction I but not in any appreciable amount in hydrolysates of 'diphosphoinositide'. Although these products may in part be derived from higher phosphoinositides, they probably arise principally from monophosphoinositide. This view is based on the finding (Dittmer & Dawson, 1961) that the monophosphoinositide of brain is separated out into the soluble phase during the precipitation procedure used in preparing 'diphosphoinositide' from fraction I.

Finally, two additional compounds were detected in mild-alkaline hydrolysates from both of these fractions. One of these migrated with inorganic phosphate on paper ionophoresis and the second, although its mobility was slightly less than that of inorganic phosphate, was only poorly separated from it. The combined fraction which migrated with inorganic phosphate, when eluted from the paper strip, was found, after correction for the inorganic phosphate present, to have an inositol : phosphate molar ratio consistently near to 1 : 2, but the glycerol : inositol ratio was of the order of 0·7 : 1. These results suggested that the fraction consisted of a mixture of inositol diphosphate and a compound which contains glycerol, inositol and phosphate in a molar ratio of 1 : 1 : 2. Separation of the mixture by paper chromatography with phenol–water–acetic acid–ethanol followed by elution and analysis of the bands substantiated this. The fractions obtained had glycerol : inositol : phosphate ratios of 0·17 : 1·0 : 1·97 and 1·0 : 0·93 : 2·1 respectively. The latter compound when hydrolysed at 100° with 1N-HCl for 10 min. produced a mixture which contained the phosphorus distributed as inositol diphosphate (27%), inositol monophosphate (22%), glycerophosphate (30%) and inorganic phosphate (14%). The nature and yield of the products is consistent with what would be expected from the acid hydrolysis of glycerylphosphorylinositol monophosphate. Other possible structures cannot, however, be excluded.

Acid hydrolysis of fraction I and 'diphosphoinositide' also produced a more complex mixture than that found with triphosphoinositide. The main phosphorus-containing products produced from fraction I were inositol triphosphate, inositol diphosphate, inositol monophosphate and glycerophosphate. On the basis of what is known of the hydrolysis products of triphosphoinositide, the inositol triphosphate and part of the inositol diphosphate, inositol monophosphate and glycerophosphoric acid were formed from triphosphoinositide. It is likely, however, that glycerophosphoric acid is mainly derived from phosphatidylserine and monophosphoinositide, and the inositol monophosphate from monophosphoinositide. The molar ratio of inositol diphosphate to inositol triphosphate in these hydrolysates was much higher than that found for triphosphoinositide.

On mild acid hydrolysis, purified 'diphosphoinositide' gave a mixture of products consisting of inositol triphosphate (37% of total phosphorus), inositol diphosphate (35%), inositol monophosphate (7%) and glycerophosphate (13%). It seems possible that the additional inositol diphosphate (above that expected from triphosphoinositide hydrolysis) in these acid hydrolysates of fraction I and 'diphosphoinositide', and the compound formed on alkaline hydrolysis (probably glycerylphosphorylinositol monophosphate), are derived from a true diphosphoinositide. If this is so, it is possible from the alkaline hydrolysis products to obtain a picture of the distribution of lipids in Folch fraction I and also in the phosphoinositide after it has been purified by multiple methanol precipitations (Table 2).

Table 2. *Distribution of monophosphoinositide, diphosphoinositide, triphosphoinositide and phosphatidylserine in fraction I and purified 'diphosphoinositide' prepared by the method of Folch* (1949)

Values were derived from an examination of alkaline hydrolysis products.

	Fraction I		'Diphosphoinositide' Purified by 8 methanol precipitations		Purified by 10 methanol precipitations	
	(% of total P)	(% of total mol.)	(% of total P)	(% of total mol.)	(% of total P)	(% of total mol.)
Monophosphoinositide	11·4	14·0	—	—	7·0	13·7
Diphosphoinositide	15·1	9·2	34·8	32·2	39·4	30·0
Triphosphoinositide	16·5	6·7	42·6	25·4	37·4	24·4
Phosphatidylserine	57	70·2	22·4	41·5	16·3	32·0

It can be seen that, although such precipitations partially remove monophosphoinositide and phosphatidyl serine, the ratio between triphosphoinositide and diphosphoinositide remains virtually unchanged.

DISCUSSION

Fig. 2 shows a summary of the chemical hydrolysis products of triphosphoinositide identified and studied in this paper. On mild acid hydrolysis the triphosphoinositide fatty acids, which in the parent compound are all linked through acyl ester bonds, are converted into diglyceride, monoglyceride and fatty acids. Since a diglyceride moiety could be deacylated by the acid either after or before it was hydrolysed away from the triphosphoinositide molecule, producing monoglyceride and free fatty acids, it seems reasonable to assume that in the parent compound the fatty acids are both esterified to the glycerol. Similarly, on acid hydrolysis 70–81 % of the phosphorus is liberated in the form of inositol triphosphate, and since triphosphoinositide contains fatty acid : glycerol : inositol : phosphate in the molar ratio 2 : 1 : 1 : 3, this would imply that the vacant hydroxyl group of the diglyceride is esterified to an inositol triphosphate unit. This conclusion is confirmed by the isolation of glycerylphosphorylinositol diphosphate as a major product of the deacylation of triphosphoinositide with alkali.

Acid and alkaline degradation therefore indicate that triphosphoinositide is a diacylglycerylphosphorylinositol diphosphate (phosphatidylinositol diphosphate). Since all naturally occurring phospholipids investigated have so far possessed the α configuration (see, for example, Baer & Maurukas, 1955), it seems likely that in triphosphoinositide the inositol triphosphate is also esterified to an α hydroxyl group in the glycerol. The present studies have shown that the two isomeric inositol triphosphates isolated by Grado & Ballou (1961) from acid hydrolysates of purified 'diphosphoinositide' are almost certainly derived from triphosphoinositide. These authors have reported that the major inositol triphosphate has the structure L-myo-inositol 1:4:5(6)-triphosphate and suggest that the intact lipid is similarly substituted. If this is so, it still remains to be determined to which phosphate grouping the diglyceride moiety is esterified.

Although it is certain that alkali preferentially attacks the acyl ester bonds of triphosphoinositide, the way in which acid attacks the lipid is less clear. Since diglyceride and inositol triphosphate are major hydrolysis products, it can be assumed that the bond between them is one site of attack. It is possible for the inositol diphosphate and glycerophosphate to be formed: (1) through deacylation followed by hydrolysis of the resultant glycerylphosphorylinositol diphosphate or its monoacyl derivative to inositol tri- and di-phosphate and glycerophosphate and possibly monoglyceride; or (2) through formation of phosphatidic acid and inositol diphosphate followed by deacylation of the former with the production of glycerophosphate and free fatty acid. The results obtained do not make it possible to decide between these two possibilities.

Our examination of Folch I fractions and purified 'diphosphoinositide' preparations has shown that the successive methanol precipitations partially eliminate phosphatidylserine and phosphatidylinositol in the supernatant. Nevertheless the results fully confirm the chromatographic conclusions of Hörhammer, Wagner & Hölzl (1960) on the complexity of the purified 'diphosphoinositide' fraction of Folch (1949). Whereas the molecular proportion of phosphatidylserine (32 %) in the preparations after ten methanol precipitations may at first sight appear large, it represents only about 0·5–0·6 % of N in the preparation [Folch (1949)

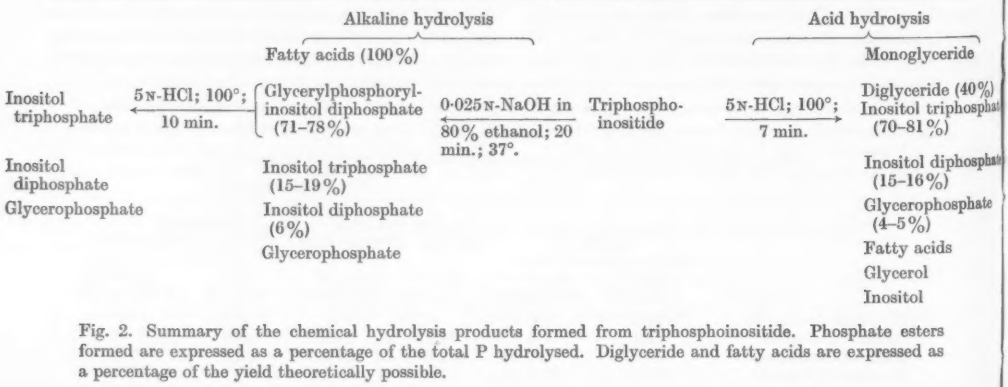

Fig. 2. Summary of the chemical hydrolysis products formed from triphosphoinositide. Phosphate esters formed are expressed as a percentage of the total P hydrolysed. Diglyceride and fatty acids are expressed as a percentage of the yield theoretically possible.

obtained 0·4–0·6% of N, 0·1–0·4% of amino N; and Grado & Ballou (1961) 0·3% of N].

On acid hydrolysis of these fractions (Folch I and purified diphosphoinositide), more inositol diphosphate is formed than can be accounted for by the triphosphoinositide present. Further, on alkaline degradation a considerable proportion of a compound is produced which is probably a glycerylphosphorylinositol monophosphate. These results suggest therefore that a true diphosphoinositide may either be present in nervous tissue or be produced from triphosphoinositide. While this compound has not yet been isolated, it is tempting to speculate from the evidence of its deacylation product that it is a phosphatidylinositol monophosphate. This would complete a series in which phosphatidylinositol (monophosphoinositide) and phosphatidylinositol diphosphate (triphosphoinositide) have already been isolated.

SUMMARY

1. Triphosphoinositide preparations isolated both from a 'diphosphoinositide' fraction of brain and from chloroform–methanol-extracted brain tissue have been hydrolytically degraded with alkali and acid.

2. Mild acid hydrolysis of triphosphoinositide produces inositol triphosphate as a major product, as well as inositol diphosphate and glycerophosphate. Diglyceride and monoglyceride have also been isolated from the reaction mixture.

3. Deacylation with alkali produces glycerylphosphorylinositol diphosphate as a major product, together with some inositol triphosphate, inositol diphosphate and a little normal and cyclic glycerophosphate. On acid hydrolysis the first compound splits, producing inositol triphosphate, inositol diphosphate and glycerophosphate.

4. From the above results it is deduced that triphosphoinositide is a diacylglycerylphosphorylinositol diphosphate (phosphatidylinositol diphosphate).

5. An examination of Folch (1949) fraction I and 'diphosphoinositide' preparations has shown that these probably contain, as well as triphosphoinositide, a true diphosphoinositide. Phosphatidylinositol and phosphatidylserine are also present.

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Interrelationships in Trace-Element Metabolism in *Aspergillus niger*

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The existence of an antagonism between heavy metals, e.g. cobalt, nickel, zinc, cadmium and copper, and essential ones, e.g. iron and magnesium, has been known for some time. The well-documented iron-deficiency 'chlorosis' in plants can be induced by high, extra-physiological concentrations of these heavy metals (Hewitt, 1951). Similar phenomena have not been studied as extensively in micro-organisms. Abelson & Aldous (1950) found that excess of cobalt, nickel, zinc, cadmium and manganese all interfered with magnesium metabolism in *Torulopsis utilis*, *Aerobacter aerogenes*, *Escherichia coli* and *Aspergillus niger*. The toxic effect on growth, with some of these organisms, could be counteracted by supplementing the culture media with magnesium. Healy, Cheng & McElroy (1955), working with *Neurospora crassa*, obtained evidence of deranged iron metabolism as

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well in metal toxicities, particularly that caused by cobalt; the decrease in the activity of iron-dependent enzymes, though not growth inhibition, could be corrected by small supplements of iron.

Recently, Sivarama Sastry, Adiga, Venkatasubramanyam & Sarma (1960*a*) have shown that, in *N. crassa*, the growth inhibition caused by cobalt and nickel toxicities can be competitively overcome by an adequate concentration of iron in the culture medium; in zinc toxicosis, iron reversed growth inhibition non-competitively. Although magnesium was also a powerful antagonist of these toxic metals, its mechanism of action was quite different from that of iron. In the light of these findings and the possible effect of metal toxicities on carbohydrate metabolism suggested by the work of Healy *et al.* (1955), interrelationships between cobalt, nickel and zinc toxicities and iron and magnesium have been investigated in the present work with the mould *A. niger*.

EXPERIMENTAL

Materials. All metal salts employed in these studies, $ZnSO_4,7H_2O$ (Merck), $CoCl_2,6H_2O$ (British Drug Houses Ltd.), $NiSO_4,6H_2O$ (May and Baker Ltd.), $MgSO_4,7H_2O$ (Merck) and ferric ammonium citrate (Fisher) were of analytical grade. The concentrations are expressed as μg. of metal/flask containing 10 ml. of medium.

Experimental organism. An acid-forming strain of *A. niger*, obtained from the National Collection of Type Cultures, Poona, India, was used. It was maintained by weekly subcultures on agar slants containing the basal medium described below with 0·1% each of malt and yeast extracts. A spore suspension in sterile, glass-distilled water, obtained from a 10-day-old culture and adjusted to 90% transmission in a photoelectric colorimeter, was used for inoculation.

Basal medium and culture conditions. A. niger was grown in 100 ml. Pyrex conical flasks, containing 10 ml. of culture medium, adjusted to pH 2·5–3·0, for 5 days at $30 \pm 1°$.

The basal medium contained (g./100 ml.): glucose, 15; KH_2PO_4, 0·25; NH_4NO_3, 0·25; $MnSO_4,4H_2O$, 0·01; $MgSO_4,7H_2O$, 0·025; $ZnSO_4,7H_2O$, 0·00065.

It was essential to use solutions made up in glass-distilled water, and to clean all glassware with chromic acid followed by thorough washing with glass-distilled water, in order to obtain consistent and reproducible results.

All experiments were repeated at least four times, and the average values have been given in the results presented below.

Assay procedures. The mycelia were harvested after 5 days' growth, washed extensively with distilled water to remove salts and spores, dried at 60–80° overnight and weighed.

Total acid produced was estimated by titrating portions (2 ml.) of culture medium, at the end of the experiment, against 0·05 N-NaOH with bromothymol blue as indicator (Sivarama Sastry & Sarma, 1957). Citric acid was analysed in suitable portions of deproteinized culture fluid by the method of Saffran & Denstedt (1948).

Glucose utilization was determined by following its disappearance from the medium, by the method of Morris (1948) as modified by Trevelyan & Harrison (1952) with anthrone. Recovery experiments showed that interference from medium constituents other than glucose was negligible at the dilutions employed.

In all the figures presented, growth, acid production and glucose-utilization values have been plotted as percentage of control values (i.e. values reached in each case with unsupplemented basal medium given above) against metal concentration (μg. of metal/flask).

RESULTS

In Figs. 1–3 are presented the effect of increasing toxic concentrations of Zn, Co and Ni respectively on growth, acid production and glucose utilization by *A. niger*. These results show that Ni is the most toxic, followed by Zn and Co, in that order. Nickel (400 μg.), Zn (1000 μg.) or Co (1200 μg.) give a growth inhibition of about 60%. Cobalt and Ni decrease growth throughout the concentration range, whereas, with up to 600 μg. of Zn, the decrease is somewhat gradual and thereafter becomes much more pronounced. Since, of the three metals, Zn is the only one that is normally needed by *A. niger* for growth as well as acid production (Steinberg, 1919; Shu & Johnson, 1948; Tomlinson, Campbell & Trussel, 1950; Nicholas, 1952), a greater tolerance of *A. niger* to excess of Zn, as shown by Fig. 1, is not unexpected.

Acid production, calculated on the basis of acid accumulated per 100 mg. of mycelial weight produced, exhibits a rapid and continuous fall with increase of Co and Ni. Zinc in amounts up to 250 μg. does not have an adverse influence in this

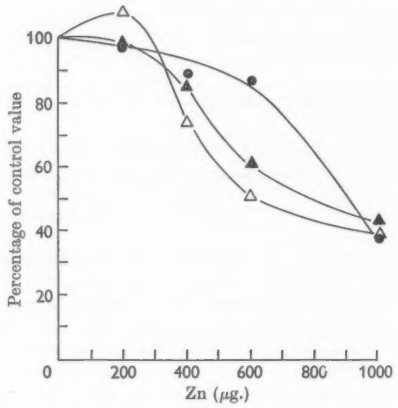

Fig. 1. Effect of zinc toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

respect; on the other hand, a consistent, though small, increase in acid has always been obtained with 200 μg. of Zn. Further, estimations of citric acid have shown that the citric acid values run in parallel with total acid. When the relative effects of Co, Ni and Zn are compared, however, it becomes evident that Ni is most inhibitory towards acid production, since with 100 μg. of Ni (Fig. 3) the inhibition in acid formation is about 62 % even when the corresponding decrease in growth is only 12 %. Neither Co nor Zn has such a pronounced influence under comparable conditions (Figs. 1, 2).

Glucose utilization, on the basis of mg. of glucose used/100 mg. of mycelial weight produced, has also been plotted as a percentage of the control values in Figs. 1–3. These Figures reveal that the extent of utilization of the carbon source is also impaired concomitantly with growth in all metal toxicities. At concentrations of the metals producing over 50 % decrease in growth, the glucose consumption does not exceed the decrease in growth. These values, which are about 50 % of control, i.e. 250–300 mg. of glucose/100 mg. of dry mycelium under the present experimental conditions, may represent the minimal rates of utilization of glucose for synthesis of cell material.

The effect of supplementation of the culture medium with Mg at various concentrations was studied in detail as regards its ability to counteract inhibition in growth, acid production and glucose utilization. In all subsequent experiments, *A. niger* was grown with 400 μg. of Ni, 1000 μg. of Zn or 1200 μg. of Co, which produced severe toxicity. These concentrations have been chosen, since, under the experimental conditions employed, they produce comparable extents of inhibition of growth. The effect of Mg supplementation was studied in the range 0–2000 μg. All values given in Figs. 4–6 have been depicted on the basis of response to supplemented Mg. The values corresponding to 'zero Mg' in these Figures are obtained in the toxic state on the basal medium alone, with the normal Mg content (246·8 μg. of Mg/10 ml.).

Magnesium is most effective with Zn, 200 μg. of additional Mg restoring growth to 94 % of the control; thereafter the growth curves reach a plateau (Fig. 4). In Ni and Co toxicities, the effect of Mg is similar except that much higher amounts (600 μg. for Ni and 2000 μg. for Co) are needed for comparable reversal (Fig. 4). If the ratio of total

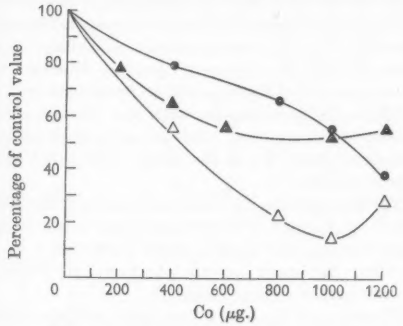

Fig. 2. Effect of cobalt toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

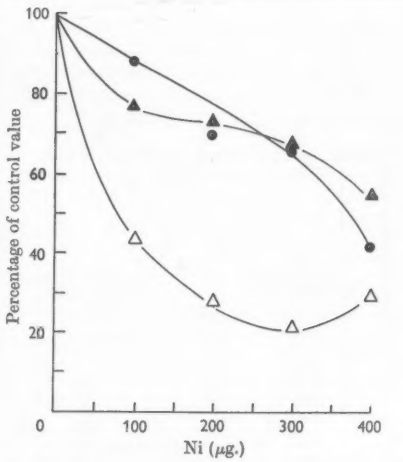

Fig. 3. Effect of nickel toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

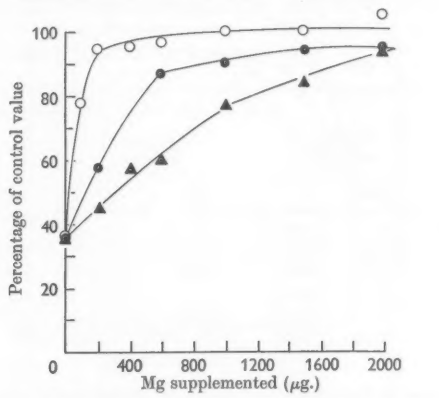

Fig. 4. Influence of magnesium on growth of *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

Mg in the medium to metal required to give an ultimate growth of 90 % of control is calculated for the three different toxicities, the values are 0·45 for Zn, 1·7 for Co and 2·1 for Ni.

The influence of Mg on acid production in the metal toxicities is shown in Fig. 5. Restoration of acid formation to normal occurs only in Zn and Ni toxicities; the small adverse effect of Mg in Co toxicity, even at a concentration of 2000 μg., where growth is normal, is not significant.

The data presented in Fig. 6 showing the influence of Mg on glucose utilization in metal toxicities, and those of Figs. 4 and 5, again indicate that complete counteraction by Mg occurs only with the metabolic derangements caused by Zn and Ni and that Mg is only partially effective in Co toxicity. Even with Zn and Ni, much higher concentrations of Mg than those required to restore growth to normal are needed to bring acid and glucose values to control levels, indicating that the effect on the latter two parameters is not necessarily only associated with increase in growth.

In view of our earlier findings that Fe can also suppress metal toxicities in *N. crassa* (Sivarama Sastry *et al.* 1960*a*), competitively in Co and Ni toxicities and non-competitively in Zn toxicity, this aspect was investigated in *A. niger*. Iron, like Mg, counteracts completely the growth inhibition by Zn, Co and Ni (Fig. 7). However, if the growth curves are compared with the corresponding ones with Mg (Fig. 4), the quantitative difference between Fe and Mg becomes obvious. Whereas the concentrations of Mg required to reverse growth retardation differ markedly with the metal toxicity concerned, in striking contrast, a uniformly high concentration of Fe in the range 1600–2000 μg. is always needed.

Fig. 8 shows the influence of Fe on glucose utilization by *A. niger* in metal toxicities. A significant improvement due to Fe takes place only in Co toxicity. Moreover, in no instance does Fe alter the amount of acid accumulated.

The effect of Mg and Fe on citric acid formation by *A. niger* in metal toxicities has not been investigated throughout the concentration range employed here. However, a few experiments were

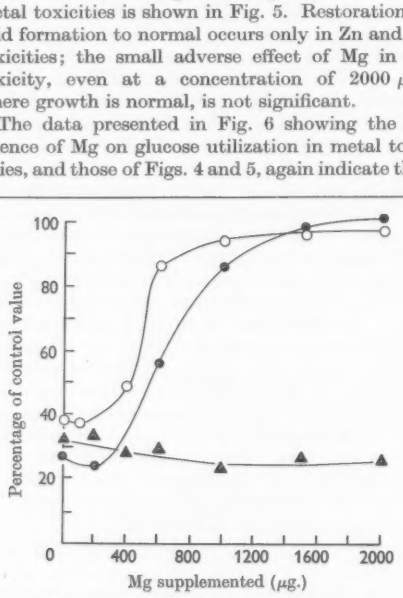

Fig. 5. Influence of magnesium on acid production by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

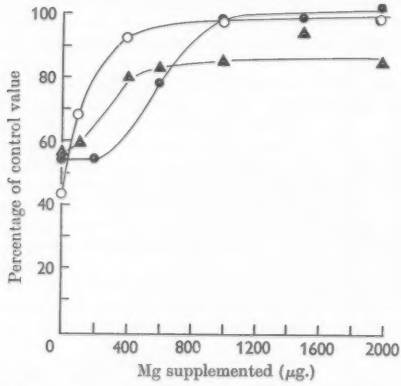

Fig. 6. Influence of magnesium on glucose utilization by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

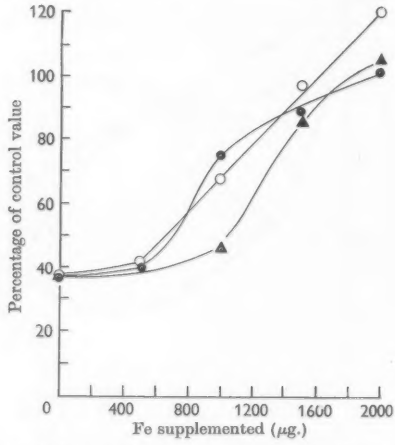

Fig. 7. Influence of iron on growth of *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

carried out to examine whether both total acid and citric acid values were restored to normal under conditions of reversal of toxicities. Results of one such typical experiment are presented in Table 1.

The absolute values for mycelial weights, total acid production and glucose utilization are also recorded. These data serve to indicate that Mg does, in fact, restore citric acid values concomitantly with those for total acid.

Lastly, a fundamental difference between Fe and Mg reversal of metal toxicities deserves mention. In all cases, only in Mg-supplemented cultures, normal sporulation occurred when growth was enhanced, the extent of sporulation running almost parallel with the improvement in growth. However, even when Fe restored growth completely to normal, there was little, if any, spore formation.

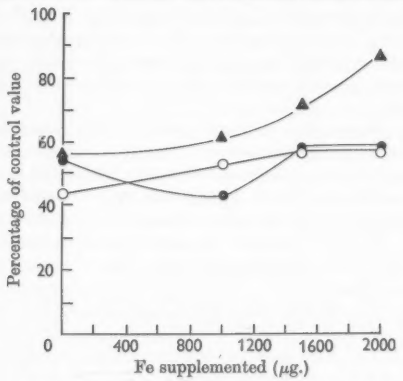

Fig. 8. Influence of iron on glucose utilization by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

DISCUSSION

The principal features of the present investigation are the marked differences not only in the quantitative aspects of cobalt, nickel and zinc toxicities in *A. niger*, but also the variations in the patterns of counteraction of these metal toxicities by iron and magnesium. Iron and magnesium show a similarity only in reversal of growth inhibition. Whereas iron restores growth values to normal, it has no effect on inhibition of acid production and is only partially beneficial in glucose utilization by *A. niger* in cobalt toxicity, being practically ineffective in this respect in the other toxicities studied. The fact that iron by itself depresses acid production by *A. niger* has been recognized by earlier workers (Tomlinson *et al.* 1950), and hence the absence of an increase in acid yields concomitantly with enhancement of growth by iron is not unexpected.

Since therefore both iron and magnesium can counteract growth inhibition, without necessarily influencing the rate of carbohydrate utilization, a reversal of metal toxicities with respect to growth, even when complete, need not inevitably involve a concomitant reversal of other metabolic derangements. This is strikingly illustrated by the response in acid-production values in metal toxicities to magnesium (Fig. 5). In cobalt toxicity, in contrast with nickel and zinc toxicities, wherein magnesium restores both growth and acid production to normal values, magnesium is completely without effect on the latter process.

In *N. crassa*, Healy *et al.* (1955) have observed that iron, at concentrations at which it has little influence on the retardation of growth due to cobalt toxicity, partially restores the activity of some of the iron-dependent enzymes. Much

Table 1. *Effects of iron and magnesium on metal toxicities in* Aspergillus niger

Acid produced was expressed as ml. of 0·05 N-NaOH required/2 ml. of culture medium. For all experimental details see text. Supplements when added were (mg.): Zn, 1·0; Co, 1·2; Ni, 0·4; Mg, 2; Fe, 2.

Supplements to 10 ml. of basal medium	Mycelial dry wt.		Acid produced		Citric acid formed		Glucose consumed	
	mg.	Percentage of control	Total	Percentage of control	mg./100 mg. of mycelium	Percentage of control	mg./100 mg. of mycelium	Percentage of control
None (control)	195	100·0	22·1	100·0	100·0	100·0	468·0	100·0
Zn	79	40·5	2·0	9·0	2·8	2·8	252·0	54·0
Zn + Mg	197	101·0	22·2	102·0	180·0	180·0	459·0	98·0
Zn + Fe	231	118·5	1·4	6·3	2·8	2·8	271·0	58·0
Co	72	37·0	1·9	8·6	3·2	3·2	264·0	56·0
Co + Mg	196	100·0	3·9	17·7	18·0	18·0	355·0	76·0
Co + Fe	198	102·0	3·6	16·3	2·7	2·7	419·0	89·0
Ni	72	37·0	2·1	9·5	4·4	4·4	284·3	61·0
Ni + Mg	190	98·0	18·6	84·5	154·0	154·0	494·0	105·5
Ni + Fe	224	115·0	2·7	12·2	10·3	10·3	299·5	64·0

higher concentrations of iron are necessary to correct the growth inhibition in *N. crassa* (Sivarama Sastry *et al.* 1960*a*). The effects of iron and magnesium in *A. niger* suggest that similar marked differences prevail as regards reversal of the metabolic derangements. In conditions when growth is restored to normal without concomitant reversal of enzymic or metabolic changes, the organism may possibly achieve normal rates of synthesis or degradation of vital metabolites with the limited amounts of the enzymes available.

The nature of the effects of magnesium and iron in suppression of metal toxicities in *A. niger* appears to be different. If, as suggested by Abelson & Aldous (1950), the primary influence of magnesium is a control of toxic-metal uptake, which presumably results in the depression of the intracellular concentrations of the toxic ions to concentrations at which they become non-toxic, it would be natural to expect all adverse influences of metal toxicities to be simultaneously annulled. Since this does not happen in cobalt toxicity (Figs. 4, 5), the control of ion uptake alone is not perhaps the only mechanism involved in the reversal of metal toxicities by magnesium.

The quantitative aspects of magnesium reversal (Fig. 4) show that, at a fairly low ratio of magnesium: zinc (0·45:1), the growth of the mould is normal. The corresponding ratios are considerably higher for cobalt and nickel. Further, in cobalt toxicity alone (Fig. 5) magnesium has no influence on acid production. From these results it is probable that zinc toxicity is largely equivalent to a conditioned magnesium deficiency, whereas the picture in cobalt toxicity indicates an iron deficiency. Such a conclusion is further justified by the fact that iron enhances the rate of glucose utilization only in cobalt toxicity (Fig. 8).

The effects of iron on nickel and zinc toxicities (Figs. 7, 8) are probably attributable to a suppression of toxic-metal accumulation at intracellular sites controlling growth, rather than to antagonism at a metabolic level. In equine erythrocytes, for example, Fe^{3+} ions have been shown to be a powerful inhibitor of ^{65}Zn uptake (Sivarama Sastry, Viswanathan, Ramaiah & Sarma, 1960*b*).

In any event, the multiplicity of metabolic derangements observed and the fact that iron and magnesium show marked differences both qualitatively and quantitatively suggests that a large number of cellular functions are impaired and that intracellular concentrations affecting each process are probably quite different. The presence of full sporulation in cultures whose growth is restored to normal by supplemented magnesium and the absence of such an effect with iron are also in line with such a possibility. The observed responses to iron and magnesium at the various concentrations studied may indicate controlling effects at different intracellular loci.

SUMMARY

1. Cobalt, nickel and zinc toxicities have been studied in detail in the mould *Aspergillus niger* from the point of view of their adverse influence on growth, acid formation and overall glucose utilization. At toxic concentrations, all these parameters were impaired to different degrees at various metal concentrations.

2. The counteracting effect of iron and magnesium when supplemented with the toxic metals has been examined. Either of these metals could overcome growth inhibition completely, iron at concentrations uniformly in the range 160–200 μg./ml. of culture medium, and magnesium at concentrations varying markedly with the metal toxicity involved. Only magnesium supplementation permitted normal sporulation concomitantly with growth.

3. Magnesium restored growth, acid formation and glucose utilization to normal levels in all cases except in cobalt toxicity, where it had no influence on acid formation and promoted glucose consumption only to the extent of about 87 % of the control. Iron reversed the impaired glucose utilization partially in cobalt toxicity alone. In all toxicities it was ineffective with regard to acid production.

4. The results have been discussed, and it has been suggested that zinc toxicity corresponded to a conditioned magnesium deficiency and that due to cobalt to an iron deficiency.

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Techniques for the Fractionation of Microbiologically Active Peptides Derived from Casein

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Hearfield & Phillips (1961) reported that the rate of growth of a strain of *Streptococcus equisimilis* in a medium based on acid-hydrolysed casein was markedly enhanced by the addition of a small amount of an enzymic digest of casein.

It seemed desirable to investigate more fully the complex mixture of peptides in such digests of casein and to establish the respective contributions made to the biological activity of digests by individual peptides.

In the past, investigations of this type were hampered by the extreme difficulty in separating peptides, first from amino acids and secondly from one another. This paper contains a description of techniques which have been successfully applied to the resolution of the peptides of casein digests and, in addition, some results on the distribution of growth-stimulating activity among the peptide fractions.

EXPERIMENTAL

Materials. Calcium caseinate was supplied by Glaxo Laboratories Ltd., acid-hydrolysed casein by Oxo Ltd., 'slab yeast' by The Distillers Co. Ltd., Sephadex G-25 by Pharmacia, Uppsala, Sweden, and Hyflo Supercel by Johns Manville and Co. Ltd.

Organism. The test organism, which was a strain of *Streptococcus equisimilis* (CN 2688 in The Wellcome Research Laboratories' Culture Collection) was freeze-dried and stored in ampoules until required.

Crude ox trypsin. This was prepared by the method of Cole & Onslow (1916). The solution obtained by this means had a content of total solids of about 3% (w/v). From its method of preparation the product almost certainly contained chymotrypsin and certain peptidases in addition to trypsin. For simplicity of expression, however, digests of casein prepared by the use of this extract will be referred to as 'trypsinized casein'.

Yeast extract. An aqueous suspension of 'slab yeast' (25%, w/v) was boiled for 30 min. Cell debris was removed by filtration with kieselguhr as filter-aid and the filtrate was then sterilized by passing it through a Ford SB pad. The resulting extract had a content of total nitrogen of about 0·1% (w/v).

Inoculum medium. The inoculum medium was of the following composition: yeast extract, 2·5 ml.; acid-hydrolysed casein, 2·25 g.; L-cystine, 1·5 mg.; DL-tryptophan, 1·0 mg.; sodium bicarbonate, 0·2 g.; thioglycollic acid, 0·05 ml.; glucose, 0·2 g.; cold tap water to 100 ml. After adjustment to pH 7·6 with 10N-sodium hydroxide the medium was sterilized by filtration.

Assay medium. The assay medium differed from the inoculum medium in containing 12·25 ml. of yeast extract, 3·0 g. of glucose and in addition 1·25 ml. of a solution (0·1%) of phenol red.

'Trypsinized casein'. Trypsinized casein was prepared by a modification of the method of Gladstone & Fildes (1940). An aqueous slurry containing calcium caseinate (10%, w/v) and sodium carbonate (0·37%) was stirred in a closed container for 4 hr. Crude ox trypsin (0·6%) and chloroform (about 1%) were then added and the temperature of the digest was raised to 37°. The pH value of the mixture, which fell during the early stages of digestion, was adjusted to 7·5 at intervals of 20 min. by the addition of 10N-sodium hydroxide. The digestion was allowed to continue at 37° with constant stirring for a total of 72 hr. After boiling for 10 min., insoluble material was removed by filtration, Hyflo Supercel being used as filter aid. The filtrate was autoclaved at 15 lb./in.^{2} for 20 min. and stored at 0° until required. The digest had a content of total nitrogen of about 1·3% (w/v) and a content of total solids of about 10% (w/v).

Preliminary fractionation of casein digests. A glass tube (250 mm. × 39 mm.), fitted at its lower end with a porous plate, was packed with a slurry of Sephadex G-25 (50 g.) in 0·1M-phosphate buffer, pH 6·8. The exact composition and even the pH of this buffer seemed to be of no special significance. As the manufacturers suggested, however, even dispersions of Sephadex were most readily produced in solutions containing inorganic salts. After the column had been allowed to settle, the top surface was covered with a disk of filter paper and the phosphate buffer displaced from the column with aq. 0·2M-ammonia solution.

The sample (2·5 ml.) of trypsinized casein was applied to the top surface of the column, allowed to soak in and then washed through with aq. 0·2M-ammonia solution. The effluent from the column was collected automatically in fractions (40 × 8 ml.) at a rate of 10 fractions/hr. Ammonia solution was used in this procedure as it is readily removed from fractions and hence does not interfere with subsequent treatment of separated materials.

Location of peptides in fractions from columns of Sephadex. In preliminary experiments, peptides and free amino acids were detected by treatment with ninhydrin after chromatographic separation on paper with butan-1-ol–acetic acid–water (Woiwod, 1949) as solvent. Subsequently it was found more convenient to examine column effluents by the following two methods.

(*a*) By determining the extinction of each fraction at 280 mμ. This indicated the distribution among the various fractions of free and combined aromatic amino acids, and yielded quickly and easily a permanent record of the column run. This was useful for comparison of different digests and for checking the reproducibility of columns.

(*b*) In the procedure to be described, the content of total amino acid in each fraction was determined colorimetrically after hydrolysis with alkali. A portion (0·025 ml.) of each fraction was pipetted into water (1·0 ml.) in a test tube and 7·5N-sodium hydroxide solution (0·3 ml.) was added. The mixture was heated at 100° for 2·5 hr. and, after cooling, 5N-acetic acid (1·0 ml.) was added. The following reagents prepared as described by Yemm & Cocking (1955) were then added in the order given: 0·2M-citrate buffer, pH 5·0 (0·5 ml.), 5% solution of ninhydrin in 2-methoxyethanol (0·2 ml.), 0·2M-potassium cyanide in 2-methoxyethanol (1·0 ml.). After mixing, the solutions were heated at 100° for 15 min. and cooled for a further 15 min. in running tap water.

The resultant purple solutions were diluted with aq. 60% ethanol (to 10 ml.) and the extinction of each solution was compared with that of a reagent blank with a Hilger Biochem absorptiometer and a filter with peak transmission at about 580 mμ. By reference to a calibration curve prepared with glycine, the results were expressed as total amino acid (mM).

Analysis of fractions from Sephadex columns by 'fingerprinting'. Peptide fractions from column runs were evaporated to dryness *in vacuo* and weighed. The solid was then dissolved in sufficient water to make a solution containing 250 mg. of solid/ml. A portion of this solution was applied to a sheet of Whatman 3 mm paper marked out as for two-dimensional chromatography. When a paper was loaded with a solution at the concentration referred to above, the end of a capillary containing the sample was applied to the paper and the liquid allowed to run out until a spot of diameter 1 cm. was formed. The spot was dried in warm air and a further application of sample made to give a spot of the same size. After drying, the sample was fractionated by descending chromatography with butan-1-ol–acetic acid–water (75 ml./sheet) as solvent. After 24 hr. the sheet was allowed to dry overnight at room temperature and the edge of the paper which had been in the chromatographic trough was trimmed off to yield a sheet of width 39 cm. The paper was then laid on a clean bench with the line occupied by the partially separated peptides along a glass rod of diameter about 2 cm. and buffer solution allowed to flow from a large hypodermic syringe on to the paper first on one side of the sample line and then on the other. It was convenient to make this application of buffer at a distance of 1 cm. from each side of the sample line. After the two buffer edges had met along the whole length of the sample line, the remainder of the paper was dipped into the buffer and blotted, care being taken not to blot the sample.

The sheet was then hung in a Perspex tank, a simplified form of the apparatus described by Katz, Dreyer & Anfinsen (1959), with the sample line horizontal and at the lower end of the paper. The upper end of the paper passed over a glass rod into a Perspex trough containing buffer solution, and the lower end of the paper dipped into a layer of buffer solution at the bottom of the tank. To help in dissipating the heat arising from the electrophoresis, the apparatus was filled completely with colourless domestic-grade paraffin. A potential difference of 2500v was then applied to the paper by means of a platinum anode, in the trough of buffer, and a platinum cathode, in the buffer layer at the bottom of the tank. To minimize the effects of changes in pH in the vicinity of the cathode, it was enclosed in a cellophan bag. With phosphate buffer, pH 7·7, *I* 0·05 (Green, 1933), duration of the electrophoretic separation was 1 hr. In this time, the current rose from about 100 to 130 mA and the temperature of the paraffin at the top of the tank rose 15–20°. At the end of the run, the paper was removed from the tank, dried at 110° and sprayed with a solution of ninhydrin and collidine in chloroform (Woiwod, 1960). On heating at 110° for about 10 min. many peptide spots became visible. Further spots developed on storing the paper overnight in polythene bags at about 4°.

Estimation of growth-stimulating activity of peptide fractions. The contents of an ampoule of dried culture were suspended in nutrient broth and streaked on blood–agar slopes. After incubating overnight at 37° a loopful of organisms was transferred from a slope into the inoculum medium (10 ml.) and kept at 37° for 8 hr. A loopful of the resulting culture was transferred into fresh inoculum medium (10 ml.), which was kept at room temperature for 9 hr., then heated to 37° in a water bath and maintained at that temperature (±0·05°) for a further 7 hr. Portions (0·1 ml.) of the culture were then used to inoculate the test media. The latter comprised portions (10 ml.) of the assay medium in 150 mm. × 15 mm. test tubes to which peptide fractions had been added. As controls, it was customary to inoculate both the basal assay medium and the assay medium fortified with 0·5% (w/v) of trypsinized casein.

Measurements of growth were made at intervals of 30 min. by determining the extinction of each culture in a Hilger Biochem absorptiometer with a filter with peak transmission at 680 mμ. Acid produced by the organism was neutralized with 0·2N-sodium hydroxide immediately before these measurements.

RESULTS

Fractionation of casein digests on columns of Sephadex. Filtration of digests through Sephadex led to a fractionation of the type illustrated in Fig. 1. Thus four groups of components were detected by the ninhydrin colorimetric procedure whereas three groups of substances were detected by measurements of ultraviolet absorption. Examination of the fractions by paper chromatography showed that tubes 10–14 contained complex material, as was evident from its streaky behaviour on chromatograms and feeble reaction with ninhydrin.

Tubes 15–20 contained peptides which chromatographed fairly well, and tubes 21–32 contained simple amino acids. Aspartic acid and glutamic acid were somewhat anomalous in their behaviour as they emerged at about tube 18.

The aromatic amino acids phenylalanine, tryptophan and tyrosine, which were very much retarded on the column, formed a peak at tube 27.

The pattern of fractionation was reproducible with a single batch of Sephadex. On comparison of batches, however, some variation was noticed, particularly in the degree of separation achieved between free amino acids and peptides of intermediate size.

Tests for growth-stimulating activity. The technique used for the preparation of the inoculum for the tests led to the early onset of rapid growth. Thus it was normally necessary to make additions of alkali within the first 3·0–3·5 hr. after inoculation, and an increase in extinction was often detectable within 1 hr. The rate of growth of the organism after 6–7 hr. in a medium containing 0·5 % of trypsinized casein was about five times as great as that in the basal medium alone (Fig. 2).

When groups of fractions (pooled as shown in Fig. 1; i–vi) from a column of Sephadex were tested, only the first four groups were found to be significantly active. The activity of the first fractions from the column was rather low but rose quickly to a maximum value in a region corresponding to the second ninhydrin-positive fraction. Thereafter, the activity fell away sharply to a low value in the fractions containing amino acids (cf. Fig. 1).

Separation of fractions of casein digests by 'finger-printing'. When the finger-printing procedure was applied to unfractionated digests of casein, only amino acids were detected, as individual peptides were not present at sufficiently high concentrations to be revealed by the ninhydrin reagent. The amino acids which were found were: leucine/isoleucine, tryptophan, phenylalanine, valine, methionine, tyrosine, proline, alanine, serine, glycine, threonine, aspartic acid and glutamic acid. Lysine, histidine and arginine appeared to have migrated into the buffer solution at the cathode end of the paper. As the procedure was not directly applicable to digests of casein, individual fractions from columns of Sephadex were examined. It was then found that whereas the peptides in fractions 9–14 were too complex to separate well in the buffer system employed, the intermediate peptides in fractions 15–20 were extremely well resolved.

The groups of fractions (i–vi) from Sephadex, referred to above, when subjected to the finger-printing procedure, gave rise to patterns, examples of which are illustrated in Fig. 3. As each fraction gave a characteristic pattern, it was clear that the

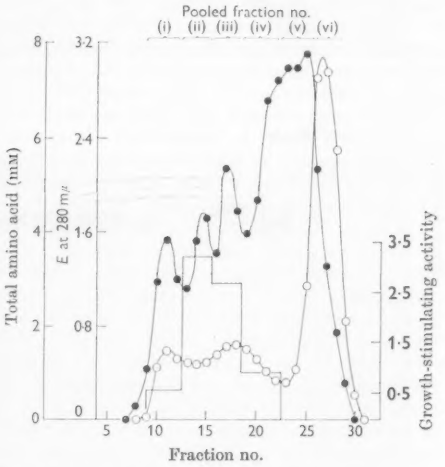

Fig. 1. Fractionation of a casein digest by filtration through Sephadex G-25. Distribution of constituents was determined by measurement of extinction at 280 mμ (○) and by the colorimetric ninhydrin procedure (●). The activities of pooled fractions (i–vi) are shown by the block figure. The values for activities were obtained from $A = (E_T - E_c)/E_c$, where E_c and E_T are respectively extinctions of cultures grown in the basal assay medium and in the assay medium to which one-quarter of each of the pooled fractions had been added (1:4). Values of A at 6, 7 and 8 hr. growth were averaged.

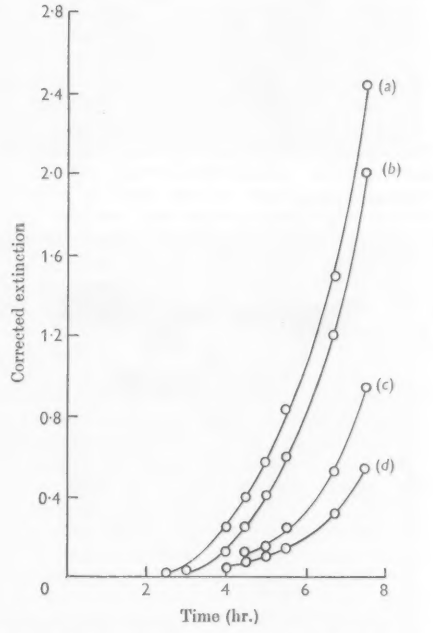

Fig. 2. Growth curves of *Streptococcus equisimilis* in assay medium containing: (*a*) 0·5 % of casein digest; (*b*) one-quarter of pooled fraction (iii); (*c*) one-quarter of pooled fraction (i); (*d*) no addition. Extinction readings were corrected for changes in volume of culture fluid arising through titration of acid formed during growth.

treatment with Sephadex had resulted in an effective separation of peptides.

The buffer referred to in the Experimental section appeared to possess a satisfactory combination of properties. Thus the ionic strength selected did not lead either to excessive heating on the one hand, or to very marked endosmotic flow on the other. Similarly, the pH value selected appeared well suited to the separation of all but the most basic compounds.

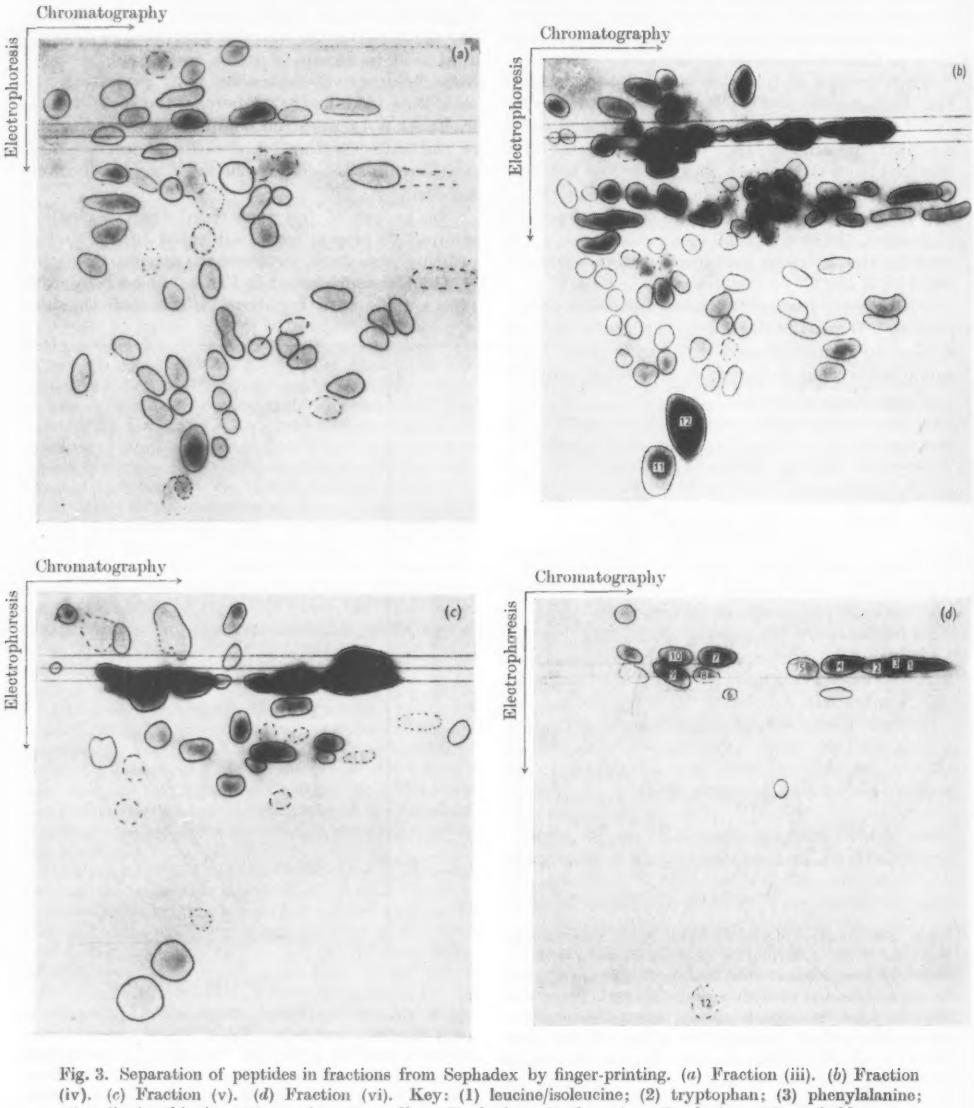

Fig. 3. Separation of peptides in fractions from Sephadex by finger-printing. (*a*) Fraction (iii). (*b*) Fraction (iv). (*c*) Fraction (v). (*d*) Fraction (vi). Key: (1) leucine/isoleucine; (2) tryptophan; (3) phenylalanine; (4) valine/methionine; (5) tyrosine; (6) proline; (7) alanine; (8) threonine; (9) glycine; (10) probably serine; (11) aspartic acid; (12) glutamic acid.

DISCUSSION

The use of paper chromatography and high-voltage electrophoresis, successively, clearly leads to the resolution of complex mixtures of peptides to a degree not possible by methods previously employed. Indeed, so detailed and reproducible are the patterns obtained that Ingram (1958) and Katz *et al.* (1959) have been able to use the technique to detect very fine differences in detailed structure between pairs of closely related proteins. It was, of course, in this context that the term 'finger-print' was first applied to the patterns obtained.

It was not feasible with trypsinized casein to use the above-mentioned method directly, owing to the low concentration of individual peptides relative to that of the free amino acids. The digest was therefore subjected to a preliminary fractionation of a type which would separate peptides from free amino acids. Such a fractionation appeared impossible by classical procedures, but recent reports by Porath (1960), that compounds of differing molecular weights may be separated from one another by filtration through the cross-linked dextran Sephadex, offered much promise. It was found that the filtration of digests through Sephadex G-25 not only gave good separation of peptides from amino acids, but the elution patterns suggested that some separation of peptides into groups had taken place. The possibility of using eluting agents other than aqueous ammonia solution was not investigated in detail, but it seems probable from observations by Porath (1960) on the separation of amino acids, peptides and proteins on columns of Sephadex that the selection of eluting agent is not critical.

Aqueous ammonia solution was convenient to use in the present instance as it conferred upon the system some measure of control of pH, was readily removed from fractions by vacuum distillation at low temperature and in addition served as a preservative when it was necessary for fractions to be stored. The presence of ammonia in the fractions did not lead to excessively high blanks in the ninhydrin colorimetric method, probably owing to the loss of ammonia which occurred during the alkaline-hydrolysis stage.

By comparison with haemoglobin, which is said to be completely excluded from the Sephadex matrix, it was concluded that the largest molecules in digests of casein were similarly completely excluded and hence passed through the column unretarded.

The aromatic amino acids, on the other hand, remained on the column until a volume of eluent had passed through which corresponded to the internal volume (V_i) of the Sephadex The value of V_i for the column was calculated from the product of the weight of Sephadex (g.) and the water-regain quoted by the manufacturers.

The value of the preliminary fractionation on Sephadex was immediately evident, as digests when treated in this way and subjected to the finger-printing procedure gave satisfactory patterns of separation. Finger-prints of individual fractions from the column in many instances showed differing patterns of peptides, confirming the impression formed earlier that the use of Sephadex led to separation of certain peptides from one another as well as from free amino acids. Whereas both of the methods referred to have separately been described by other authors, the present work, in which over one hundred different peptides have been detected in trypsinized casein, indicates that maximum usefulness may be derived from these methods by applying them successively.

Turning to the more detailed examination of fractions obtained from Sephadex, the observation that four well-defined peaks were detected by the ninhydrin method, whereas only three components were detected by measurements at 280 mμ, suggests that, in the digest, aromatic amino acids occur in combination in relatively few molecular species; the failure of the two sets of curves to show any correspondence indicates that these compounds constitute only a small proportion of the total peptide material. The observation that the bulk of the growth-stimulating activity was located in a fairly narrow region of the total effluent suggests that maximal activity is associated with peptides of particular molecular dimensions. In this connexion, Merrifield & Woolley (1958), working with *Lactobacillus casei*, failed to detect high strepogenin activity in peptides containing fewer than five amino acid residues and showed that an upper limit existed for the size of peptides with this type of activity.

Hearfield & Phillips (1961) have described the separation from trypsinized casein of factors which appeared to exert their action at different stages in the growth of the bacterium. In the present study no such effect was detected and, indeed, the fractions from Sephadex gave rise to a series of growth curves which were similar to one another, differing only in their slopes. As the fractionation on Sephadex may be regarded as a form of molecular sieving, it may be inferred that the two types of growth pattern previously referred to do not, contrary to expectation, reflect the action of peptides differing on the basis of molecular weight. Indeed, it now seems likely that fractions of a given mean molecular weight contain a balanced mixture of peptides active in both the early and later stages of growth. It therefore appears probable that the previously reported differences in bio-

logical properties of peptides derived from casein reflected more specific differences in chemical constitution.

SUMMARY

1. Columns of Sephadex G-25 have been used for the preliminary fractionation of tryptic digests of casein.

2. Fractions from Sephadex columns have been examined for their ability to stimulate the growth of a strain of *Streptococcus equisimilis*. Maximum stimulatory activity appeared to be localized in a fairly narrow region of the total peptide material.

3. Further fractionation of peptides derived from casein has been achieved by subjecting fractions from columns of Sephadex to two-dimensional separations involving the successive use of paper chromatography and high-voltage electrophoresis. Over one hundred spots due to peptides have been detected.

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The Colorimetric Reaction between Vitamin A_2 Aldehyde and Antimony Trichloride

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The occurrence of vitamin A_1 aldehyde in the eggs of some marine teleosts has recently been reported (Plack, Kon & Thompson, 1959). To extend this study to the eggs of freshwater teleosts, a method for the determination of vitamin A_2 aldehyde was required, since preliminary experiments had shown that the A_2 form was present together with vitamin A_1 aldehyde in salmon eggs (Plack, 1958). Cama *et al.* (1952) and Farrar, Hamlet, Henbest & Jones (1952) have prepared crystalline vitamin A_2 aldehyde and, for the colorimetric reaction with antimony trichloride, gave $\lambda_{max.}$ 730–740 mμ fading to 705 mμ, and 730 mμ fading to 705 mμ, respectively. Wald (1938–39) had previously found this $\lambda_{max.}$ to be 705 mμ, and Wilt (1959), with extracts from natural materials, found $\lambda_{max.}$ to be between 700 and 705 mμ. The study of the colorimetric reaction between vitamin A_2 aldehyde and antimony trichloride reported here shows how these differences of $\lambda_{max.}$ may have arisen and, though not exhaustive, provides the basis for a quantitative test.

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EXPERIMENTAL

Preparation of vitamin A_1 aldehyde. Vitamin A_1 aldehyde was prepared from the crystalline alcohol (Roche Products Ltd.) as described by Plack *et al.* (1959). For the preparation of vitamin A_2 aldehyde, the A_1 aldehyde was purified by chromatography but not crystallized.

Preparation of vitamin A_2 aldehyde. Vitamin A_1 aldehyde (2·38 g.) was converted into vitamin A_2 aldehyde as described by Henbest, Jones & Owen (1955). The crude preparation in light petroleum (b.p. 40–60°), was adsorbed on a 20 cm. × 4 cm. column of 300 g. of aluminium oxide (British Drug Houses Ltd., for chromatographic adsorption analysis) weakened by the addition of 30 ml. of water, and the column was developed with 2·5 l. of 2% (v/v) of freshly-redistilled diethyl ether in light petroleum (b.p. 40–60°). A yellow diffuse band was eluted first, followed by a strong orange band, but neither contained vitamin A_2 aldehyde. The main orange band was eluted more slowly and was collected in three fractions. From their absorption curves, fraction 1 contained much vitamin A_1 aldehyde and was rejected, but fractions 2 and 3 contained reasonably pure vitamin A_2 aldehyde and were combined; the solvent was removed under reduced pressure, and the residue dissolved in 30 ml. of light petroleum (b.p. 40–60°). Spontaneous crystallization occurred overnight at −20°, appar-

ently from one nucleus, and the yield was 0·695 g. Two recrystallizations from light petroleum were carried out, and the last traces of solvent were removed under high vacuum (0·01 mm. Hg), to give a final yield of 0·488 g. The crystals of aldehyde were sealed in 16 evacuated small glass ampoules.

The crystals were monoclinic, with m.p. 66–68° (Kofler apparatus), unchanged by further recrystallization. Both Cama *et al.* (1952) and Farrar *et al.* (1952) reported melting points of 77–78°, although one preparation (Cama *et al.*) had m.p. 61°. With the exception of $\lambda_{max.}$ in chloroform, values for the absorption characteristics of my preparation (Table 1) were in good agreement with those given by Farrar *et al.* (1952) and in fair agreement with the values obtained by Cama *et al.* (1952).

A sample of crystals of m.p. 77–79° was recently received (a gift from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland); with the Kofler apparatus its m.p. was found to be 75–77°. A solution in light petroleum of the crystals of m.p. 66–68° was seeded with the higher-melting-point material and gave crystals of m.p. 75–77°. A solution of these last crystals was cooled to −20° and seeded with material of m.p. 66–68°, when two types of crystal formation were produced: a few clusters with a purple tinge, m.p. 75–77°, and a larger quantity of orange–red clusters, m.p. 66–68°.

The vitamin A_2 aldehyde crystals of m.p. 66–68° were used in the experiments reported here, but the quantitative aspects of the reaction were checked with crystals of m.p. 75–77°.

A solution of the aldehyde in hexane was isomerized with I_2 (see Fisher, Kon & Plack, 1957). The extinction was about 5% lower, and $\lambda_{max.}$ about 1 mμ lower, after isomerization, suggesting that the original aldehyde was all-*trans* rather than a *cis*-isomer. Solutions in $CHCl_3$ of the aldehyde were stable for many weeks when kept in brown-glass containers in a dark cupboard at room temperature.

Antimony trichloride. Two preparations were used. The solution of $SbCl_3$ in $CHCl_3$ supplied by British Drug Houses Ltd. for vitamin A tests, containing about 14% (w/w) of $SbCl_3$, will be referred to as $SbCl_3$ solution (commercial). Solutions prepared by distilling solid A.R. $SbCl_3$ (British Drug Houses Ltd.) and collecting the middle fractions in a flask containing a weighed quantity of $CHCl_3$ (used as supplied by May and Baker Ltd.) will be referred to as purified $SbCl_3$ solution. Initially, the $CHCl_3$ was purified by washing with water, to remove ethanol and decompose any $COCl_2$, and drying over anhydrous Na_2SO_4. Solutions of $SbCl_3$ in this purified $CHCl_3$ frequently gave an oily deposit, which disappeared on heating but returned on cooling. Such a deposit was not produced when $CHCl_3$, as supplied, was used. These solutions prepared in the laboratory were usually diluted to contain 14% (w/w) of $SbCl_3$, but other concentrations were also tested. Acetic anhydride, A.R., when required, was added to the solutions at the rate of 2% (v/v).

For the reaction with vitamin A_2 aldehyde, 2 ml. of the reagent was rapidly pipetted into a glass cell of 1 cm. light-path containing 0·5 ml. of a solution of the aldehyde in $CHCl_3$, and the cell was quickly transferred to the spectrophotometer for measurement.

Antimony pentachloride. The diphasic system of Brüggemann, Kraus & Tiews (1952) was used, and the reagent was prepared as described by them from solid $SbCl_3$, $K_2Cr_2O_7$ and conc. HCl. One drop (about 0·05 ml.) was added to 3 ml. of the aldehyde solution in dry, ethanol-free $CHCl_3$ in a stoppered flask, the flask was shaken gently for 1 min. and the contents poured into a spectrophotometer cell for measurement. Most of the aqueous phase remained in the flask.

Solutions of commercial $SbCl_5$ (British Drug Houses Ltd.) 15, 1·5 and 0·15% (v/v) in $CHCl_3$ were also used, 1 drop together with 1 drop of acetic anhydride being added to 2 ml. of the aldehyde solution in $CHCl_3$.

Glycerol dichlorohydrin. 1:3-Dichloropropan-2-ol, for vitamin A determination, was obtained from Kodak Ltd. and used as supplied, since preliminary tests only were carried out. To 2 ml. of the reagent in a spectrophotometer cell, 0·5 ml. of the aldehyde solution in $CHCl_3$ was added, the two solutions were mixed with a Polythene rod and the cell was transferred to the instrument.

Spectrophotometers. Most measurements of light-absorption were carried out with an Optica CF4 double-beam recording spectrophotometer, equipped with a red-sensitive DuMont photomultiplier. This instrument was used in one of three ways: (*a*) to give a plot of percentage transmission against time after addition of reagent for one particular wavelength; (*b*) to give percentage transmission against wavelength; (*c*), a combination of (*a*) and (*b*), with the chart running continuously to give a time scale, and the region of maximum absorption scanned in rapid succession, so that changes of $\lambda_{max.}$ with time could be followed. Readings in terms of percentage transmissions were converted into extinction values, and in some later experiments extinction values were plotted directly.

After $\lambda_{max.}$ had been determined with the recording spectrophotometer, more exact readings of extinction were obtained with fresh samples in a Beckman model DU quartz spectrophotometer, or in the manual version of the Optica CF4 spectrophotometer.

RESULTS

Reaction with antimony trichloride

Commercial solutions and solutions of purified antimony trichloride. When antimony trichloride

Table 1. *Absorption characteristics of the preparation of vitamin A_2 aldehyde (m.p. 66–68°) compared with those of preparations obtained by other workers*

	Plack, this paper		Cama *et al.* (1952)		Farrar *et al.* (1952)	
Solvent	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$*
Chloroform	410	1280	407	1360	407	1295
Ethanol	400	1370	397	1410	400	1350
Hexane	387	1440	385†	1490†	388‡	1460‡

* Calculated from values for ε. † In light petroleum (b.p. 40–60°). ‡ In pentane.

Table 2. *Colorimetric reaction between chloroform solutions of vitamin A_2 aldehyde and purified antimony trichloride (14%): variations in $\lambda_{max.}$ with time after mixing and with concentration of aldehyde in the presence or the absence of acetic anhydride*

Vitamin A_2 aldehyde concentration in reaction mixture (μg./ml.)																			
2·83		2·27		1·70		1·13		0·566		0·283		0·141							
Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)	Time (min. sec.)	$\lambda_{max.}$ (mμ)						
With acetic anhydride																			
0 47	732	0 48	732	0 48	732	0 47	730	0 47	728	0 49	724	0 46	708						
1 33	731	1 35	730	1 24	730	1 34	727	1 28	722	1 28	710	1 43	704						
2 16	730	2 24	731	1 59	728	2 20	724	2 10	715	2 21	705	2 27	702						
3 0	728	3 13	728	2 46	727	2 55	719	2 55	711	3 8	704	3 4	702						
3 35	728	4 0	727	3 34	726	3 39	715	3 41	709	3 43	701	4 0	701						
4 17	725	4 36	726	4 10	723	4 23	713	4 27	707	4 30	701	4 46	700						
5 3	726	—	—	4 57	723	5 19	711	5 4	706	5 17	702	—	—						
Without acetic anhydride																			
0 56	742	0 54	742	0 57	741	0 57	741	0 53	740	0 51	741	0 55	740						
1 30	741	1 39	742	1 34	741	1 47	741	2 10	740	1 38	741	1 43	741						
2 5	742	2 14	742	2 11	741	2 24	741	3 0	741	2 24	742	2 27	740						
2 39	742	2 47	741	2 57	741	3 13	741	3 47	741	3 9	740	3 14	740						
3 26	742	3 21	742	3 45	741	4 9	741	4 35	741	3 57	741	3 59	740						
4 0	742	3 56	742	4 24	741	4 56	740	5 20	741	4 43	740	4 43	739						
4 35	741	4 39	741	4 57	741	—	—	—	—	—	—	—	—						

solution (commercial) with acetic anhydride was used as the reagent, solutions of vitamin A_2 aldehyde gave a green–blue colour, the $\lambda_{max.}$ of which varied with the concentration of aldehyde. In one instance, a reaction mixture containing 2·05 μg. of aldehyde/ml. had $\lambda_{max.}$ 733 mμ, and one containing 0·205 μg./ml. had $\lambda_{max.}$ 705 mμ. Similar variations were found with all batches of antimony trichloride solution (commercial), but different batches gave different values of $\lambda_{max.}$ for the same solution of aldehyde. They also required different times to reach $E_{max.}$, the variation being from 30 sec. to 2 min. 30 sec., and gave different rates of fall of $\lambda_{max.}$ with time. The colour reactions of these various batches of antimony trichloride solution (commercial) with vitamin A_1 alcohol were perfectly normal.

Antimony trichloride was therefore purified by distillation, and dissolved in chloroform. A 14% solution in the presence of acetic anhydride gave no improvement in the variations of $\lambda_{max.}$ (Table 2).

Effect of acetic anhydride. With a 14% solution of purified antimony trichloride, the effect of acetic anhydride was studied. In Table 2 are shown the values for $\lambda_{max.}$ for different concentrations of vitamin A_2 aldehyde at different times after the reagent and aldehyde solutions had been mixed. In one experiment, acetic anhydride was added to the reagent, in the other it was omitted. Only when acetic anhydride was absent were the values for $\lambda_{max.}$ constant (741 mμ; range, in this and other experiments, 739–743 mμ) irrespective of time after mixing and concentration of vitamin A_2 aldehyde. This result was reproducible with all the batches of 14% purified antimony trichloride solution prepared.

The same experiments were done with vitamin A_1 aldehyde, and, both in the presence and absence of acetic anhydride, $\lambda_{max.}$ was constant with time and with concentration of aldehyde. The two values were slightly different, however, being 661 mμ (range 659–663 mμ) with added acetic anhydride and 666 mμ (range 665–667 mμ) in its absence.

A commercial solution of antimony trichloride, with no added acetic anhydride, still gave variations in $\lambda_{max.}$ of from 738 to 730 mμ with final concentrations of vitamin A_2 aldehyde of from 1·98 to 0·198 μg./ml. Purification of the reagent and omission of acetic anhydride were therefore both necessary for stability of $\lambda_{max.}$.

Acetic anhydride had no effect on the ultraviolet curve of vitamin A_1 aldehyde or A_2 aldehyde.

Effect of concentration of antimony trichloride. Table 3 shows the effect of different concentrations of purified antimony trichloride with a single concentration of vitamin A_2 aldehyde in the absence of acetic anhydride. Values for $E^{1\%}_{1\,cm.}$ were nearly constant over the range 10–16% of antimony tri-

Table 3. *Effect of concentration of purified antimony trichloride on the colorimetric reaction with vitamin A_2 aldehyde*

The reaction mixture contained 0·5 ml. of a solution of vitamin A_2 aldehyde in $CHCl_3$ (7·16 μg./ml.) and 2 ml. of a solution of purified $SbCl_3$ in $CHCl_3$, to give a final concentration of 1·43 μg. of aldehyde/ml. $E_{max.}$ (741 mμ) was measured with the manual Optica spectrophotometer and $E^{1\%}_{1\,cm.}$ calculated from the mean of three measurements; the time factor and $\lambda_{max.}$ were determined with the Optica recording spectrophotometer.

Concn. of $SbCl_3$ solution added (%, w/w)	$E^{1\%}_{1\,cm.}$ (741 mμ)	Time after mixing when $E_{max.}$ steady (sec.)	$\lambda_{max.}$ (mμ)
5	3500	20–25	738
10	4330	20–35	742
12	4400	20–35	742
14	4400	30–45	742
16	4300	40–75	743
20	4030	50–90	743
25	3770	20–60	745
30	3840	20–45	744

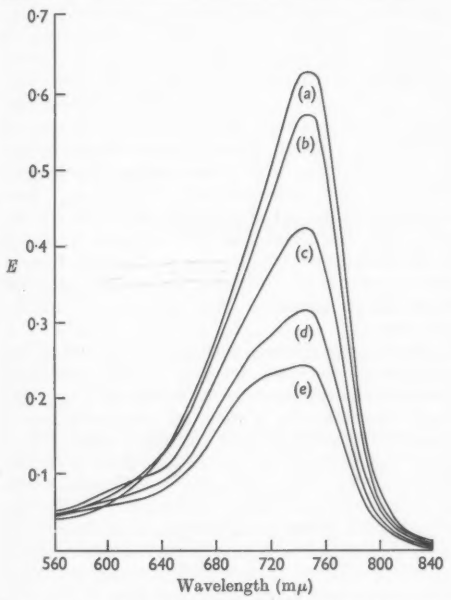

Fig. 1. Curves drawn by the recording spectrophotometer for the green–blue colour produced by the reaction of 0·5 ml. of a solution of vitamin A_2 aldehyde in chloroform (7·16 μg./ml.) and 2 ml. of a 14% solution of purified antimony trichloride in chloroform, showing the fading of the colour with time. The peak of curve (*a*) was traced 45 sec. after the reaction had begun, (*b*) 2 min. 7 sec., (*c*) 6 min. 22 sec., (*d*) 10 min. 35 sec. and (*e*) 14 min. 38 sec. Speed of scanning 8 mμ/sec.

chloride, but at concentrations above and below they were lower. Since the absorption maximum was broad, values for $E^{1\%}_{1\,cm.}$ at $\lambda_{max.}$ would not be significantly different from those given at 741 mμ. $\lambda_{max.}$ tended to increase with concentration of antimony trichloride, but was reasonably constant over the range 10–20%. The period of stability of $E_{max.}$ varied with concentration of trichloride, but the maximum stability, at concentrations of 20 and 25% (approx. 40 sec., Table 3), was associated with values of $E^{1\%}_{1\,cm.}$ below the maximum. The stability of $E_{max.}$ varied slightly with different preparations, and it is probably also dependent on the rate of mixing the reagent and the test solution, and on temperature.

At 20°, a saturated chloroform solution of antimony trichloride contains about 20% (w/w), so that the 25 and 30% solutions were supersaturated. At lower temperatures, the commercial solutions of about 14% sometimes crystallize out. It seemed sensible from the result of this experiment to use the same concentration of antimony trichloride used for other purposes, namely 14% (w/w).

Quantitative aspects of the reaction. With a 14% solution of purified antimony trichloride and no acetic anhydride, the maximum value of the extinction at 741 mμ increased linearly with concentration of vitamin A_2 aldehyde up to 10 μg./ml. (final concentration of aldehyde in reaction mixture, 2 μg./ml.). A mean value for $E^{1\%}_{1\,cm.}$ (741 mμ) of 4200 (range 4000–4400) was obtained with five preparations of antimony trichloride solution and solutions of vitamin A_2 aldehyde prepared from four separate weighings. With these same solutions, $\lambda_{max.}$ varied over the range 739–743 mμ. Solutions of the crystals of m.p. 75–77° gave values within these ranges.

Concentrated solutions of the aldehyde, m.p. 66–68°, gave an initial fleeting red colour in this reaction, which immediately changed to green–blue.

Fig. 1 shows a series of curves drawn by the recording spectrophotometer at various times after mixing chloroform solutions of vitamin A_2 aldehyde and purified antimony trichloride. They are not true absorption curves, since during the time of scanning one curve (35 sec.) the extinction diminished slightly. With the high speed of scanning used, there is also a slight displacement of $\lambda_{max.}$ to longer wavelengths. As the extinction at $\lambda_{max.}$ diminished, the shoulder at about 700 mμ became more pronounced.

Reaction with antimony pentachloride

Preliminary experiments with antimony pentachloride in a diphasic system showed that the $\lambda_{max.}$ of the green–blue colour varied from 705 to 724 mμ with increasing concentrations of vitamin A_2 aldehyde. The rate of fading was rapid and the specific

extinction coefficient not so great as with antimony trichloride. Antimony pentachloride in chloroform gave no colour reaction, though with the 15 and 1·5% (v/v) solutions the yellow colour of the aldehyde disappeared.

Reaction with glycerol dichlorohydrin

Vitamin A_2 aldehyde gave a blue–green colour with glycerol dichlorohydrin, rising to a maximum intensity about 2 min. after mixing, and starting to fade a few seconds later. With final concentrations of aldehyde in the reaction mixture of 0·27–4·33 μg./ml., $\lambda_{max.}$ of the coloured product was constant at 618 mμ. Over this range of concentrations, the colour intensity was proportional to the concentration of aldehyde, and the mean $E^{1\%}_{1\,cm.}$ (618 mμ) was 1340. Vitamin A_1 aldehyde with glycerol dichlorohydrin gave a coloured product with $\lambda_{max.}$ 653 mμ, with a shoulder at 610 mμ.

DISCUSSION

Vitamin A and its related compounds are usually determined either by measuring their absorption of light in the ultraviolet or by a colorimetric reaction, of which the Carr–Price test (Carr & Price, 1926) with antimony trichloride is the most widely used. Each method has certain advantages, but in this Laboratory we prefer the colorimetric technique, since it is more sensitive and less subject to interference when small quantities of vitamin A in extracts from natural materials are to be measured. For the determination of vitamin A_2 aldehyde, there is another reason for choosing a colorimetric method. The aim was to devise a method for the determination of vitamin A_1 and A_2 aldehydes in the presence of each other (see Plack & Kon, 1961); in the ultraviolet, the wavelengths of their respective absorption maxima vary according to the solvent used, but are separated by only about 20 mμ, whereas in the colour reaction with antimony trichloride the difference between their maxima is much greater, 75 mμ in the absence of acetic anhydride, so that the detection of the two aldehydes in mixtures is easier and the determination of each more accurate with this test.

The Carr–Price test with antimony trichloride is applied in slightly different ways in different laboratories; the amount of acetic anhydride used varies, and so does the proportion of reagent to sample; the instrument available for measuring the colour intensity may use a small or broad band width and may determine whether the maximum intensity can be read or whether a reading at some fixed time after mixing reagent and sample must be used. Extinction coefficients must therefore be determined for the conditions used, and for the purpose of defining them for this Laboratory a sample of crystalline vitamin A_2 aldehyde was prepared. Henbest *et al.* (1955) were unable to obtain crystalline material by their method, but I was fortunate enough to obtain spontaneous crystallization which gave a product of m.p. 66–68°, considerably different from the two values previously reported, 77–78° (Farrar *et al.* 1952; Cama *et al.* 1952) and 61° (Cama *et al.* 1952), and yet from its absorption characteristics apparently pure all-*trans*-vitamin A_2 aldehyde. Vitamin A_1 aldehyde is known to exist in dimorphic forms, with m.p. 57 and 65° (Robeson, Blum, Dieterle, Cawley & Baxter, 1955) and it may be that all-*trans*-vitamin A_2 aldehyde exists in three different crystalline forms.

The reactions of solutions of the crystalline aldehyde with antimony trichloride in the presence of acetic anhydride gave, even with purified materials, green–blue colours whose $\lambda_{max.}$ varied with concentration of aldehyde. This variation (Table 2) possibly accounts for the discrepancies in $\lambda_{max.}$ reported in the literature. Two other colour reactions were tested in an effort to overcome this variation. With antimony pentachloride, in a diphasic system, there was still variation of $\lambda_{max.}$ with concentration of aldehyde; antimony pentachloride in chloroform produced no colour with vitamin A_2 aldehyde, although Murata & Nagashima (1960) have recently used this reagent successfully for determination of vitamin A_1 alcohol. With glycerol dichlorohydrin $\lambda_{max.}$ did not vary with concentration of aldehyde, but vitamin A_1 aldehyde gave a higher $\lambda_{max.}$ than vitamin A_2 aldehyde and, for the latter, the specific extinction coefficient was low compared with that in the Carr–Price test; the reagent therefore seemed less suitable than antimony trichloride, provided that the irregularities with the latter could be overcome.

Further experiments with purified antimony trichloride showed that omission of acetic anhydride from the reaction mixture led to values of $\lambda_{max.}$ that were constant over a range of concentrations of vitamin A_2 aldehyde and remained stable for at least 5 min. (Table 2). Acetic anhydride is usually added in the antimony trichloride reaction to remove any traces of water and avoid clouding, but Carr & Price (1926) in their original communication do not mention its use, and in the reaction reported here it is essential to omit it. The omission did not lead to any cloudiness in the solutions.

Conditions have thus been found for a quantitative colorimetric test for vitamin A_2 aldehyde with antimony trichloride. The wavelength of maximum absorption is not affected by changes in aldehyde concentration, the colour intensity is stable for a short time, and varies linearly with the concentration of vitamin A_2 aldehyde. With the range of values for $E^{1\%}_{1\,cm.}$ (4000–4400, mean 4200)

found for different preparations of antimony trichloride, the characteristics of a particular preparation should be determined with pure vitamin A_2 aldehyde for accurate work, but for determinations of the aldehyde in natural materials, the mean value of 4200 can be used. The variations in $\lambda_{max.}$ (739–743 mμ, mean 741 mμ) are not significant since, with the broad maximum of the absorption curve, values of extinction over this range would be essentially the same, within the experimental error of the test.

SUMMARY

1. Crystalline vitamin A_2 aldehyde with m.p. 66–68° has been prepared from vitamin A_1 aldehyde.

2. With purified antimony trichloride, 14 % (w/w) in chloroform containing 2 % (v/v) acetic anhydride, the wavelength of maximum absorption of the green–blue colour produced with vitamin A_2 aldehyde increased with concentration of the aldehyde and decreased with time.

3. In the absence of acetic anhydride, the wavelength of maximum absorption of the colour product was steady at 741 mμ, the colour developed remained at full intensity for about 15 sec., the maximum extinction varied linearly with concentration of aldehyde and $E^{1\%}_{1\,cm.}$ (741 mμ) was 4200 (range 4000–4400).

4. Some observations on antimony pentachloride and glycerol dichlorohydrin (1:3-dichloropropan-2-ol) as colorimetric reagents for vitamin A_2 aldehyde are presented.

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A Comparative Survey of the Distribution of Vitamin A Aldehyde in Eggs

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We have recently reported the occurrence of vitamin A_1 aldehyde in the ripe eggs of several marine teleosts (Plack, Kon & Thompson, 1959) and in hen's eggs (Plack, 1960). Pollard & Bieri (1959) confirmed generally our early findings, but found vitamin A_2 aldehyde in the eggs of American 'herring' from Chesapeake Bay, and Winterstein & Hegedüs (1960) confirmed our finding with hen's eggs. With cod, the aldehyde is found only in eggs from ripening ovaries (Plack, Woodhead & Woodhead, 1961) and from our experience it is so with other fish.

Some preliminary experiments with ripe eggs from salmon suggested that vitamin A_2 aldehyde was present (Plack, 1958), but a study of the eggs of freshwater and anadromous fish was held up for lack of a suitable method of determining vitamin A_1 and A_2 aldehydes in the presence of each other. The two aldehydes can be determined by a simple extension of the colorimetric reactions of the two compounds with antimony trichloride, but this method could only be applied accurately when the anomalous behaviour of vitamin A_2 aldehyde in the colorimetric reaction had been clarified by Plack (1961).

With the method now available, a comparative survey of the occurrence of vitamin A_1 and A_2 aldehydes in eggs was made. The aldehydes were found in all the ripe vertebrate eggs so far examined, but not in eggs from five invertebrates.

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MATERIALS AND METHODS

Chemicals

Solvents. Solvents, of A.R. quality whenever available, were used as supplied by May and Baker Ltd., except that diethyl ether, 'peroxide free', of which recent batches were found to be contaminated with a substance that combines with vitamin A aldehydes, was kept over NaOH pellets overnight and carefully redistilled before use, the middle fractions only being collected. The contaminant is removed by this procedure. Hexane of spectroscopic grade was obtained from Hopkin and Williams Ltd.

Antimony trichloride. For the determination of vitamin A_1 and A_2 aldehydes, A.R. $SbCl_3$ crystals were purified as described by Plack (1961) and dissolved in $CHCl_3$ to give a 14% (w/w) solution. For the determination of vitamin A_1 and A_2 esters and alcohols, a solution of $SbCl_3$ in $CHCl_3$, for vitamin A tests, supplied by British Drug Houses Ltd. was used.

Vitamin A compounds. Vitamin A_1 aldehyde was prepared as described by Plack *et al.* (1959) and vitamin A_2 aldehyde as described by Plack (1961). Vitamin A_1 alcohol was purchased from Roche Products Ltd. and vitamin A_2 alcohol, m.p. 63–65°, was a gift from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland.

Collection and storage of biological materials

Invertebrates. Egg cases of the cockroach, *Periplaneta americana* L., were provided by the D.S.I.R. Pest Infestation Laboratory, Slough, Bucks., and those of the African migratory locust, *Locusta migratoria migratorioides* R. & F., by the Anti-Locust Research Centre, London, S.W. 7. The lipids were extracted from these two samples on arrival. From the lobster, *Homarus vulgaris* Milne Edwards, eggs, which were developing but had not reached the 'eyed' stage, were collected for us by Mr G. Davies, Fisheries Experiment Station, Conway. Dr L. R. Fisher collected ovaries from the escallop, *Pecten maximus* (L.) and ovaries from the sea urchin, *Echinus esculentus* L., were obtained from the Marine Station, Millport; these two samples and the lobster eggs were stored in deep-freeze at −25°.

Cephalochordate. Specimens of *Amphioxus lanceolatus* (Pallas) were obtained from the Laboratory of the Marine Biological Association of the United Kingdom, Plymouth, and ovaries were removed and stored in deep-freeze until sufficient material was available.

Vertebrates. (*a*) Lampreys. Specimens of the lampern, *Lampetra fluviatilis* (L.) and the sea lamprey, *Petromyzon marinus* L., were purchased from Mr P. J. Gaskins, New Haw Farm, Tirley. Ovaries from 30 lampern were bulked and analysed as one sample, and ovaries from 8 sea lampreys were similarly treated. Both samples were stored in deep-freeze before analysis.

(*b*) Marine elasmobranchs. Ovaries from marine elasmobranchs were collected for us by Dr B. B. Rae, Marine Laboratory, Aberdeen, and stored in deep-freeze. Table 1 gives information about the ovaries analysed, and in all samples eggs of various degrees of ripeness were present.

(*c*) Marine teleosts. Deep-frozen herring were purchased locally. Plaice ovaries were collected by Mr A. B. Bowers, Marine Biology Station, Port Erin, Isle of Man, and ovaries of the other marine teleosts by Dr B. B. Rae; all were stored in deep-freeze. Table 2 gives particulars of the material analysed.

(*d*) Freshwater teleosts and salmon. The ovaries of the carp (*Cyprinus carpio* L.) and the inactive ovaries of the pike (*Esox lucius* L.) were collected in September 1958 from the Neusiedler See in Burgenland, Austria, by one of us (S. K. K.), and a sample of ripe pike eggs stripped from the fish was sent to us frozen from the same locality by Dr P. Schubert. Salmon (*Salmo salar* L.) eggs were supplied by Dr G. Howells, Freshwater Fisheries Laboratory, London, and eggs or ovaries from char [*Salvelinus alpinus willughbii* (Günther)], perch (*Perca fluviatilis* L.), pike (*Esox lucius* L.) and trout (*Salmo trutta* L.) were collected by Mr

Table 1. *Particulars of the elasmobranch ovaries analysed*

Species	Date caught	Length of fish (cm.)	Weight of ovary (g.)	No. and size of eggs
Black-mouth dogfish, *Pristiurus melanostomus* (Rafin.)	21 June 1959	63	5·5	7 eggs 0·5–0·7 cm. diameter and smaller eggs
Rabbit fish, *Chimaera monstrosa* L.*	23 June 1959	—	54·5	5 eggs 2·0 cm. diameter and smaller eggs
	24 June 1959	—	113	About 12 eggs 1·0–2·0 cm. diameter and smaller eggs
Spur dog, *Squalus acanthias* L.	26 Sept. 1958	92	194†	7 eggs 3·5–4·0 cm. diameter and smaller eggs
	26 Sept. 1958	89	190†	6 eggs 3·4–4·2 cm. diameter and smaller eggs
	19 June 1959	—	214	Probably 6–7 large eggs and smaller eggs, rather disintegrated
Thornback ray, *Raia clavata* L.	25 June 1959	71	40·1	5 eggs 1·0–3·0 cm. diameter and smaller eggs
	26 Sept. 1959	71, 72	233‡	About 50 eggs 1·3–2·0 cm. diameter and smaller eggs

* Subclass Holocephali.
† Does not include weight of embryos, which were discarded.
‡ Combined weight of ovaries from two fish.

Table 2. *Particulars of the samples of ovaries from marine teleosts*

Species	Date caught	No. of pairs of ovaries in sample	Weight of sample (g.)
Coelorhynchus coelorhynchus (Risso)	23 June 1959	2	32·1
	24 June 1959	2	20·6
Grey gurnard, *Trigla gurnardus* L.	26 June 1959	3	36·8
Herring, *Clupea harengus* L.	Oct.–Nov. 1960	1	40·3
		1	39·5
		1	27·3
		1	32·4
John Dory, *Zeus faber* L. (spent)	29 June 1959	1	8·3
(ripe)	26 June 1959	1	55·7
Plaice, *Pleuronectes platessa* L.	2 Feb. 1959	1	156
	12 Feb. 1959	4	219
	12 Feb. 1959	4	301
Red gurnard, *Trigla cuculus* L.	25 June 1959	1	62·6
	26 June 1959	2	119

G. J. Thompson, Freshwater Biological Association, Windermere. All these eggs or ovaries were stored in deep-freeze and, with the exception of the pike ovaries from Austria, all were ripe.

(*e*) Reptiles. Adders, *Vipera berus* (L.), killed in the New Forest were collected by Mr G. N. Gould and stored at 0° until sent to Shinfield. Ovaries were then dissected out and stored in deep-freeze. Eggs 0·5–1·5 cm. long from adders caught in May 1960 were analysed. Four eggs from a European water tortoise, *Emys orbicularis* (L.), and two eggs from a South American terrapin (species not known) were obtained for us from the Bristol Zoo by Miss V. M. Kon and six terrapin eggs [from a mixed tank containing *Pelusios subniger* (Lacépède), *Geoemyda punctularia* (Daudin) and *Pseudemys scripta* (Schoepff)] were received from Dr H. G. Vevers, Zoological Society of London. These eggs were stored in deep-freeze. Fresh turtle eggs [probably from *Chelonia mydas* (L.)] were the gift of Mr A. C. Lusty of William Lusty Ltd. They were flown from Mombasa and stored in a refrigerator for 2 days before analysis.

(*f*) Amphibian. Six female common frogs, *Rana temporaria* L., were purchased from L. Haig and Co. Ltd., Newdigate, Surrey, at the end of February 1961. They were pithed and the eggs removed, mixed and used immediately.

(*g*) Bird. Eggs from the domestic hen, *Gallus gallus* L. (Rhode Island Red × Light Sussex) were obtained from one of the Institute's breeding stocks. The diet contained 200 μg. of added vitamin A/100 g. and 5% (w/w) of dried grass, but was deficient in vitamin B_{12}.

Extraction of lipids

The sample was made as homogeneous as possible. For this purpose ovary tissue was blended if sufficient was available, otherwise it was cut into small pieces and mixed. Egg cases of insects were cut in half before extraction. Egg shells of the water tortoise, terrapins and turtle were removed and the whites and yolks extracted together. The yolks only of hen's eggs were extracted.

Method A. A portion of the sample, usually 30 g., was freeze-dried in a centrifugal freeze-drier of the type described by Record & Taylor (1958). The dried sample was weighed, ground to a powder, transferred to a Soxhlet thimble and extracted with 150 ml. of diethyl ether for 4 hr. The residue from the thimble was then transferred to a Waring Blendor, blended with 200 ml. of light petroleum (b.p. 40–60°), and 60 ml. of ethanol and 30 ml. of water were added. Blending was continued for 2 min. and the light-petroleum layer poured off. The aqueous residue was extracted twice more with 200 ml. lots of light petroleum and the three extracts were combined. Any solid matter in the diethyl ether and light-petroleum extracts was allowed to settle and the supernatant extracts were evaporated to dryness in clean flasks on a steam bath, the last portions of solvent being removed under reduced pressure. The residues were weighed and dissolved in hexane, and the solutions made to suitable volumes (usually 12 ml.) with hexane.

Method B. A portion of the sample, usually 30 g., was homogenized in lots of about 10 g., each with 7·5 ml. of water, in a Potter–Elvehjem homogenizer with a nylon pestle. The homogenates, together with one washing of the homogenizer with 7·5 ml. of water, were transferred to a Waring Blendor, blended with 200 ml. of light petroleum (b.p. 40–60°); 60 ml. of ethanol was then added, and the whole was blended for a further 2 min. The procedure of method *A* was then followed. When the sample contained much connective tissue, homogenization was not possible and a portion of the sample was treated directly in the Waring Blendor.

Determination of dry-matter content

Dry matter was determined by drying about 5 g. of the sample to constant weight in an oven at 105°.

Determination of vitamin A_1 and A_2 aldehydes

Separation of the aldehydes from vitamin A esters and alcohol. The method of separation described by Plack *et al.* (1959) was applied to the light-petroleum extract obtained by method *A*, or to the whole extract obtained by method *B*. Usually one-third of the extract was used, but not more than 0·5 g. of lipid was put on the column. Aluminium oxide, for chromatographic adsorption analysis (British Drug Houses Ltd.), was later substituted for alumina from Peter Spence and Sons Ltd. in the separation and, after

drying at 150°, was weakened by the addition of 12% of its weight of water. Vitamin A_1 and A_2 aldehydes were not separated by this method.

The diethyl ether extracts obtained from frog's and hen's eggs and sea-urchin ovaries by method *A* were saponified by a method similar to that described by Winterstein & Hegedüs (1960) before separation of the aldehydes. To each 1 g. of lipid, 50 ml. of diethyl ether and 5 ml. of 10% (w/v) KOH in methanol were added, and the mixture was left at room temperature overnight in an atmosphere of N_2. The mixture was then washed with 3×20 ml. of water, the remaining diethyl ether solution was dried over anhydrous Na_2SO_4 and evaporated to dryness, and the residue dissolved in hexane. The recovery of vitamin A_1 aldehyde after this mild saponification was about 80%, so that the content of aldehyde in the diethyl ether extracts could not be determined accurately.

Portions of the diethyl ether extract from the rabbit fish, thornback ray, grey gurnard, red gurnard and adder were tested for aldehyde by the chromatographic method without this mild saponification.

Carr–Price test. The two aldehydes were determined by the Carr–Price colorimetric reaction. An Optica CF4 double-beam recording spectrophotometer fitted with a red-sensitive DuMont photomultiplier was used to measure the intensity of the blue colour while a stream of N_2 was passed through the cell compartments. The spectrophotometer was set up at 665 mμ with $CHCl_3$ in the control cell and 0·5 ml. of $CHCl_3$ and 2 ml. of purified $SbCl_3$ solution in $CHCl_3$ in the stoppered experimental cell, and the base-line over the range 600–840 mμ was recorded. The instrument was reset to 665 mμ. To the sample, 0·5 ml., in the experimental cell was added 2 ml. of purified $SbCl_3$ solution from a bulb pipette and the cell was transferred to the instrument. The chart drive was switched on 20 sec. after addition of the $SbCl_3$ solution had begun, and the chart kept running at a speed of 2 in./min. The percentage transmission, or extinction, was recorded at 665 mμ for 10 sec., then at 740 mμ for 10 sec. and then alternately at 665 and 740 mμ until the maximum extinctions at the two wavelengths had been reached, usually between 20 and 80 sec. after mixing. A single value for the extinction at 840 mμ was then recorded, and with the wavelength drive on to give a scanning speed of 1·25 mμ/sec. a curve of the light-absorption of the blue colour product was drawn. The curve is not a true absorption curve, since the blue colour is fading during the scanning, but it does give the wavelengths of absorption maxima and of inflexions with reasonable accuracy, as they are scanned in a few seconds. The wavelengths of 665 and 740 mμ chosen are both 1 mμ below the maxima for vitamin A_1 and A_2 aldehyde in this test (Plack, 1961) but were used for convenience in changing the spectrophotometer rapidly from one wavelength to the other. At 840 mμ there should be no significant absorption of light due to the colour products of vitamin A_1 and A_2 aldehydes.

The procedure of reading the extinctions of the colour product alternately at 665 and 740 mμ could be carried out on a Beckman DU spectrophotometer although the curve could not be traced. Readings were then obtained at intervals of 15–20 sec., so that 30–40 sec. elapsed between successive readings at one wavelength. During this time the maximum extinction may have been missed, but the error involved was small.

Calculation of results. The method is based on the simple additive effect of the colour product of vitamin A_1 and A_2 aldehydes. At 740 mμ, the extinction of the colour product of vitamin A_1 aldehyde was $0{\cdot}01\,E_{665\,m\mu}$; at 665 m$\mu$, the extinction of the colour product of vitamin A_2 aldehyde was $0{\cdot}38\,E_{740\,m\mu}$. In the absence of acetic anhydride $E^{1\%}_{1\,cm.}$ (666 mμ) = 4150 was found for the colour product of vitamin A_1 aldehyde, and $E^{1\%}_{1\,cm.}$ (741 mμ) = 4200 for the colour product of vitamin A_2 aldehyde was found by Plack (1961). At 665 and 740 mμ, the two extinction coefficients did not differ significantly from the values given above for 666 and 741 mμ.

If the curve of the colour product was of the normal form, i.e. peaks or inflexions at about 665 and 740 mμ, with the curve falling off to low extinctions on each side, the extinctions at 665 mμ due to vitamin A_1 aldehyde (x) and at 740 mμ due to vitamin A_2 aldehyde (y) were calculated from the simultaneous equations:

$$x + 0{\cdot}38y = E_{665\,m\mu} - E_{840\,m\mu},$$

$$0{\cdot}01x + y = E_{740\,m\mu} - E_{840\,m\mu},$$

the maximum extinctions found at 665 and 740 mμ being corrected for the small extinction at 840 mμ, which was regarded as due to general background absorption. The concentrations of vitamin A_1 and A_2 aldehydes were then calculated from the values of the specific extinction coefficients given above.

Mixtures containing as little as 5% of vitamin A_1 aldehyde and 95% of vitamin A_2 aldehyde showed the presence of the former by an inflexion at about 683 mμ on the curve of the Carr–Price colour product. Mixtures of 95% A_1 and 5% A_2 aldehydes did not show a definite inflexion of the curve in the region of 740 mμ, but when the proportion of vitamin A_2 aldehyde reached 10% an inflexion was found.

Proof of the presence of vitamin A_2 aldehyde. The sample of pike eggs from Austria was chosen for further tests, since it contained a high proportion of vitamin A_2 aldehyde and there was sufficient material available. Method *B* was used to extract 60 g. of eggs, the extract in hexane was divided into six parts and the aldehyde separated from each by gradient elution as described above. Those fractions with $E_{370\,m\mu} > 0{\cdot}015$ were put together, the solvent was removed, and 10 ml. of spectroscopic-grade hexane was added for the determination of $\lambda_{max.}$ in the ultraviolet. The hexane was then removed under reduced pressure and 8 ml. of ethanol were added to the mixture. Not all the lipid dissolved, and the supernatant liquid was poured off from the residue. A few milligrams of $NaBH_4$ were added to the solution, which was then shaken. After about 5 min., when the yellow colour had disappeared, the solution was transferred to a separating funnel and 20 ml. of light petroleum and 8 ml. of water were added to extract the vitamin A_2 alcohol formed by reduction together with the other lipids. Two further extractions with 20 ml. quantities of light petroleum followed, the combined extracts were washed with 3×20 ml. of water and dried over anhydrous Na_2SO_4, the solvent was removed under reduced pressure, and about 3 ml. of hexane was added. Chromatography on alumina followed (see above), and the eluate obtained with 8% (v/v) ethanol in hexane was dried under reduced pressure, and the residue dissolved in 8 ml. of ethanol. $\lambda_{max.}$ in the ultraviolet and in the Carr–Price test (in $CHCl_3$ solution) were determined with the recording spectrophotometer.

To 1·5 ml. of this ethanol solution, 1·5 ml. of a 0·04 N-solution of HCl in ethanol was added and consecutive absorption curves were traced with the recording spectrophotometer to follow the formation of ethoxyanhydrovitamin A_1 (Henbest, Jones, Owen & Thaller, 1955).

Determination of vitamin A_1 and A_2 esters and alcohols

Purification by chromatography and saponification. The separation of vitamin A esters and alcohol in the two extracts obtained by method *A* and in the extract obtained by method *B* was carried out by the chromatographic method of Thompson, Ganguly & Kon (1949) with the modification of Kon, McGillivray & Thompson (1955). In general one-third of the total extract was used for this separation, but not more than 0·5 g. of lipid was put on the column. The two fractions were saponified and extracted as described by Plack (1956) except that aqueous 60% (w/w) KOH solution was used. Each fraction was subjected to a further chromatography, usually as described by Thompson *et al.* (1949), and the eluates containing vitamin A, now in the alcohol form, were dried and $CHCl_3$, 1·5 ml. or more, was added for the Carr–Price test.

With the frog's and hen's eggs, the final chromatography after saponification was replaced by a gradient elution chromatography to separate vitamin A alcohol from xanthophylls. The apparatus described by Plack *et al.* (1959) was used, with 20% (v/v) diethyl ether in light petroleum in the bottle and diethyl ether in the separating funnel. The non-saponifiable residue in hexane was put on the column and elution begun. The first 40 ml. of eluate contained β-carotene, if present, and was discarded; the next 100 ml. contained vitamin A alcohol, and the residue after removal of solvent was taken up in $CHCl_3$; the xanthophylls remained on the column.

In all these procedures, vitamins A_1 and A_2 remained together.

Carr–Price test. The determination of vitamin A_1 and A_2 alcohols by the Carr–Price reaction was similar to that already described for vitamin A_1 and A_2 aldehydes but, with the alcohols, the maximum intensity of the blue colour is reached a few seconds after addition of the reagent, and the recording spectrophotometer could not be used. The direct-reading instrument described by Thompson (1949) was therefore used. To 0·5 ml. of the $CHCl_3$ solution of vitamin A in the cell 1 drop of acetic anhydride was added followed by 2 ml. of the commercial solution of $SbCl_3$ in $CHCl_3$. The maximum intensity of the blue colour was read first at 620 mμ and afterwards with another 0·5 ml. of the $CHCl_3$ solution, at 693 mμ. No correction for possible background absorption could be made with this instrument (see above).

Under these conditions, a value of $E^{1\%}_{1\,cm.}$ (620 mμ) = 5000 was found for vitamin A_1 alcohol (see Fisher, Kon & Thompson, 1956) and the ratio $E_{620\,m\mu}/E_{693\,m\mu}$ was 0·01. For vitamin A_2 alcohol, a value of $E^{1\%}_{1\,cm.}$ (693 mμ) = 3520 was found, and the ratio $E_{620\,m\mu}/E_{693\,m\mu}$ was 0·42. With mixtures of the pure compounds, the extinctions due to the two components at 620 and 693 mμ were found to be additive, and x, the extinction at 620 mμ due to vitamin A_1 alcohol, and y, the extinction at 693 mμ due to vitamin A_2 alcohol, could be calculated from the equations:

$$x + 0{\cdot}42y = E_{620\,m\mu},$$

$$0{\cdot}01x + y = E_{693\,m\mu}.$$

The concentrations of the two components were then calculated from the specific extinction coefficients given above. In the results, the concentrations of vitamin A_1 and A_2 esters and alcohols in the extracts have been added together, the results being expressed in terms of vitamin A_1 alcohol; the small difference in molecular weight between vitamin A_1 and A_2 alcohols has been ignored.

Estimation of carotenoids

A rough estimate of the total carotenoids present in a sample was obtained by diluting a portion of the extract with an appropriate volume of hexane, centrifuging to clear if necessary, and determining the absorption curve over the range 380–540 mμ with the recording spectrophotometer. A value for $E^{1\%}_{1\,cm.}$ of 2500 was used to calculate the quantity of carotenoids present from the extinction of the highest peak. Some extracts were yellow in colour but either did not show the characteristic peaks of the carotenoid pigments, or showed them only as inflexions on a curve, the extinction of which decreased steadily with increase in wavelength. The small amounts of carotenoids in such extracts, if present, are reported as not measurable.

RESULTS

Table 3 shows the results of the determination of total vitamin A_1 and A_2 aldehydes and the percentage of vitamin A_2 aldehyde in solutions of

Table 3. *Total vitamin A_1 and A_2 aldehydes and percentage of vitamin A_2 aldehyde determined in mixtures of known composition*

Determinations were by the Carr–Price test (see Methods).

Composition of mixture		Total vitamin A aldehyde found (as percentage of theoretical)	Percentage of vitamin A_2 aldehyde found (as percentage of theoretical)
Vitamin A_1 aldehyde (μg./ml.)	Vitamin A_2 aldehyde (μg./ml.)		
4·64	0·51	101·0	97·0
4·12	1·02	101·4	97·9
3·09	2·05	101·0	100·0
2·06	3·07	99·2	100·8
1·03	4·10	99·9	100·4
0·51	4·61	101·9	101·0

known composition containing mixtures of the pure compounds. Table 4 shows similar results for vitamin A_1 and A_2 alcohols. The two methods gave satisfactory results with the pure substances.

Ovaries of the sea urchin were extracted by method *A* and eggs or ovaries of the cockroach, locust, lobster and escallop by method *B*. No vitamin A was found in the eggs or ovaries of these animals and the cockroach eggs contained no carotenoids, but eggs of the locust and lobster, and ovaries of the escallop and sea urchin, contained 60·1, 498, 64·5 and 18·6 μg. of carotenoids/g. dry matter respectively. Ovaries of *Amphioxus lanceolatus*, a cephalochordate, were extracted by method *B* and contained 0·23 μg. of vitamin A aldehyde and 0·26 μg. of total vitamin A esters and alcohol/g. wet wt., the vitamin A_2 forms not being determined.

Tables 5–8 give the results of the other analyses. All the ripe eggs, or ovaries containing ripe eggs, of the vertebrates contained vitamin A aldehyde and the ester and alcohol forms of vitamin A.

Table 4. *Total vitamin A_1 and A_2 alcohols and percentage of vitamin A_2 alcohol determined in mixtures of known composition*

Determinations were by the Carr–Price test (see Methods).

Composition of mixture		Total vitamin A alcohol found (as percentage of theoretical)	Percentage of vitamin A_2 alcohol found (as percentage of theoretical)
Vitamin A_1 alcohol (μg./ml.)	Vitamin A_2 alcohol (μg./ml.)		
9·00	1·00	98·6	101·0
8·00	2·00	100·2	99·0
6·00	4·00	97·9	102·0
4·00	6·00	98·7	101·3
2·00	8·00	99·1	100·6
1·00	9·00	97·5	101·1

Table 5. *Mean concentrations of vitamin A and carotenoids in the eggs of lampreys and marine elasmobranchs*

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. All samples were ovaries, and were extracted by method *A*. N.M., Not measurable.

Species	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids (μg./g.)
	Total (μg./g.)	Percentage of A_2	Total (μg./g.)	Percentage of A_2	
Lampern (1)	0·37	21	22·0	22	7·3
Sea lamprey (1)	0·08	—	2·10*	49*	N.M.
Black-mouthed dogfish (1)	1·31	10	2·24	11	N.M.
Rabbit fish (2)	1·02	8	1·29*	17*	N.M.
Spur dog (3)	2·02	10	8·84	14	N.M.
Thornback ray (2)	0·80	21	0·88*	51*	5·0

* See p. 567.

Table 6. *Mean concentrations of vitamin A and carotenoids in the eggs of marine teleosts*

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. All samples were ovaries. N.M., Not measurable.

Species	Method of extraction	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids (μg./g.)
		Total (μg./g.)	Percentage of A_2	Total (μg./g.)	Percentage of A_2	
Coelorhynchus coelorhynchus (2)	*A*	14·10	4	4·66	33	19·3
Grey gurnard (1)	*A*	2·95	14	2·10	23	13·3
Herring (4)	*B*	3·73	13	1·33*	50*	N.M.
John Dory (spent) (1)	*B*	0	—	1·66*	52*	18·4
(ripe) (1)	*B*	3·07	9	1·03*	—	24·2
Plaice (3)	*B*	5·30	16	0·47*	—	—
Red gurnard (2)	*A*, *B*	3·46	18	0·95*	46*	9·4

* See p. 567.

Amongst the lampreys and marine elasmobranch fish (Table 5) the concentration of vitamin A aldehyde in the ovaries was about equal to, or less than, the concentration of esters + alcohol; vitamin A_1 was predominant, and small amounts of carotenoids were present. In the eggs of marine teleosts (Table 6), vitamin A_1 was again predominant, but the concentration of aldehyde was equal to or greater than the concentration of esters + alcohol, and substantial amounts of carotenoids were usually present. With the exception of those of the carp and the char, the eggs of the freshwater teleosts and the anadromous salmon (Table 7) contained predominantly vitamin A_2, and in general the concentration of aldehyde was equal to or slightly greater than the concentration of esters + alcohol; with the exception of the perch, substantial amounts of carotenoids were present. Eggs of the amphibian, the reptiles and the bird (Table 8) contained predominantly vitamin A_1, the concentration of the aldehyde form was about equal to or less than that of the esters + alcohol, and substantial amounts of carotenoids were present in the eggs of all animals except those of the adder. With the latter some 24% of the total aldehyde was present in the diethyl ether extract.

The high values for the proportion of vitamin A_2 esters and alcohols in the eggs of certain marine elasmobranchs and teleosts are possibly erroneous, owing to the accumulation of experimental errors and the small concentrations of esters and alcohols present (see Discussion).

Evidence for the presence of vitamin A_1 and A_2 aldehydes was usually obtained by their chromatographic behaviour and the $\lambda_{max.}$ of their colour products in the Carr–Price test. With the extract of ripe pike eggs from Austria, further experiments were carried out to establish the presence of vitamin A_2 aldehyde. The vitamin A aldehydes were separated from the esters and alcohols and $\lambda_{max.}$ in hexane was 389 mμ; in the Carr–Price test $\lambda_{max.}$ was 743 mμ. Reduction with sodium borohydride gave $\lambda_{max.}$ in ethanol 355 mμ, and in the Carr–Price test 691 mμ, both close to the values for

Table 7. *Mean concentrations of vitamin A and carotenoids in the eggs of freshwater teleosts and the salmon*

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. N.M., Not measurable.

Species	Ovaries (O) or eggs (E) analysed	Method of extraction	Vitamin A aldehyde: Total (μg./g.)	Vitamin A aldehyde: Percentage of A_2	Vitamin A esters + alcohol: Total (μg./g.)	Vitamin A esters + alcohol: Percentage of A_2	Carotenoids (μg./g.)
Carp (1)	O	*B*	6·25	34	7·45	52	75·2
Char (3)	E	*A*	3·47	37	1·83	54	18·1
Perch (6)	4O, 2E	*B*	5·97	97	3·38	88	N.M.
Pike (Austria) (1)	E	*B*	4·94	96	4·14	98	10·3
(2) (inactive ovaries)	O	*B*	0	—	1·70	94	11·0
Pike (England) (6)	4O, 2E	*B*	3·78	90	2·40	86	8·8
Salmon (4)	E	*A*	3·75	72	2·05	64	16·9
Trout (5)	E	2*A*, 3*B*	2·89	80	3·66	73	24·2

Table 8. *Mean weight and vitamin A and carotenoid content of the eggs of an amphibian, some reptiles and a bird*

Numbers of samples analysed are shown in parentheses. All samples were extracted by method *A*. N.M., Not measurable.

Species	Weight of egg (g.)	Vitamin A aldehyde: Total (μg./egg)	Vitamin A aldehyde: Percentage of A_2	Vitamin A esters + alcohol: Total (μg./egg)	Vitamin A esters + alcohol: Percentage of A_2	Carotenoids (μg./egg)
Amphibian						
Frog (1)	—	1·72*	11	2·74*	27	35·0*
Reptile						
Adder (1)	—	1·00†	4	0·77†	14	N.M.
European water tortoise (1)	7·7	3·25	13	6·19	14	20·6
South American terrapin (2)	30·8	8·71	18	8·59	34	280
Terrapin (1)	7·5	2·96	14	5·32	11	47·5
Turtle (8)	46·6	6·80	4	12·1	—	91·6
Bird						
Domestic hen (2)	69·7	24·3	4	131	10	885

* μg./g. dry matter. † μg./g. wet wt.

vitamin A_2 alcohol given by Farrar, Hamlet, Henbest & Jones (1952). Treatment of the reduced product with ethanolic hydrogen chloride gave a substance with $\lambda_{max.}$ 353, 370 and 391 mμ in ethanol, corresponding to ethoxyanhydrovitamin A_1 (Henbest *et al.* 1955). Vitamin A_2 aldehyde was therefore the predominant form in the extract.

DISCUSSION

Methods of extraction

The two-stage method of extraction, in which the sample is freeze-dried, first extracted with diethyl ether and the residue extracted with light petroleum in the presence of ethanol and water, was introduced to cope with the large proportion of lipid present in the yolk of hen's eggs, which interferes with the determination of vitamin A aldehyde (Plack, 1960). The diethyl ether extraction removed 75–80 % of the lipid of these eggs but little or no aldehyde, which was extracted with the remainder of the lipid by light petroleum in the presence of ethanol and water. This method has been used with other eggs known to contain much lipid, or known to give anomalous results in the determination of vitamin A aldehyde. Such anomalous behaviour, for instance a rapidly rising extinction at 665 mμ, was not found after a preliminary freeze-drying and Soxhlet extraction with diethyl ether had removed some of the lipid. We have tested chromatographically the diethyl ether extracts from two samples of marine teleost ovaries and two of elasmobranch ovaries, which contained only small quantities of fat, and could find no definite proof of the presence of aldehyde; application of the mild-saponification method to the diethyl ether extract from frog's and hen's eggs revealed no aldehyde, and a trace, respectively. With the eggs of the adder, and herring ovaries (P. A. Plack, unpublished observations), some aldehyde was present in the diethyl ether extracts, and was probably the 'free', as opposed to the combined, aldehyde reported previously (Plack *et al.* 1959). This 'free' aldehyde is possibly an artifact produced under certain storage conditions. The adders were kept for some days at 0° before the ovaries were removed and frozen, and the herring, obtained from commercial sources, probably had the same treatment.

The combined aldehyde, left behind in the solid residue after diethyl ether extraction, was mostly broken down by the extraction with light petroleum in the presence of ethanol and water, so that the light-petroleum extract contained largely 'free' aldehyde and a small amount of combined aldehyde, from which 'free' aldehyde was later liberated by chromatography through water-weakened alumina.

The second extraction method involves breaking the small fish eggs in a Potter–Elvehjem homogenizer and extracting with light petroleum in the presence of ethanol. By this means all the aldehyde and the lipids are extracted together. With this second method the total vitamin A was divided into four parts, vitamin A_1 and A_2 esters and alcohols, whereas, after the two-stage extraction, vitamin A_1 and A_2 esters and alcohols were determined separately in the diethyl ether and light-petroleum extracts, so that eight separate values had to be added to give the total for esters and alcohol.

We have previously reported (Plack *et al.* 1959) that although chloroform–methanol mixtures extract more lipid from herring eggs, the yield of aldehyde is similar to that obtained by method *B*.

From the experience gained in the course of this study, we would suggest that for the extraction of eggs the two-stage method *A* should be used, but the diethyl ether extract should be tested for aldehyde, after a mild saponification, in addition to the light-petroleum extract, and portions of the two extracts should be combined, saponified and chromatographed for the determination together of the ester and alcohol forms of vitamin A.

Determination of vitamin A_1 and A_2 aldehydes

The behaviour of vitamin A_2 aldehyde in the Carr–Price test has been described by Plack (1961), and the method described here is based on his findings. Tests of the method with solutions containing mixtures of pure vitamin A_1 and A_2 aldehydes have given satisfactory results (Table 3); but at lower concentrations in extracts from natural materials, in which the magnitude of the extinction at 840 mμ may be similar to that of the extinctions at 665 and 740 mμ from which it is subtracted, the determination of the two forms of aldehyde is likely to be less precise.

A similar method for the determination of vitamin A_1 and A_2 aldehydes in frog's eyes has been described by Wilt (1959), but under his conditions vitamin A_2 aldehyde showed a maximum extinction at 705 mμ in the Carr–Price test. The equations used in the two methods are therefore not comparable.

Chloroform solutions of crystalline vitamin A_1 and A_2 aldehydes are stable for many weeks when kept in amber flasks in a dark cupboard at room temperature, and samples of an extract of pike's eggs, stored in hexane at 4° in the dark, gave similar values for the concentration of total vitamin A aldehyde over a period of 5 days, the lowest value being 97 % of the highest (P. A. Plack, unpublished observations). It seems likely, therefore, that little loss of aldehyde would have occurred during the one or two days between extraction and determination.

Determination of vitamin A_1 and A_2 esters and alcohols

Many workers (e.g. Cama, Collins & Morton, 1951) have used the Carr–Price test to determine vitamin A_1 and A_2 alcohols in mixtures, and the method is described here merely to indicate the conditions and the factors used.

Schwieter (1960) has recently crystallized vitamin A_2 alcohol and a sample of the all-*trans* form was given to us by F. Hoffmann–La Roche and Co. Ltd. In the Carr–Price test, $\lambda_{max.}$ was 693 mμ, in agreement with the findings of Farrar *et al.* (1952) and Cama & Morton (1953), but $E^{1\%}_{1\,cm.}$ (693 mμ) was 3520, lower than the values of 4000 and 3870 reported by these authors. The difference is no doubt due in part to the different conditions under which the test was carried out. Cama & Morton (1953) gave a value of 0·409 for the ratio $E_{620m\mu}/E_{693m\mu}$, and the value used by us (0·42) is similar. Values for this ratio tended to vary slightly, but variations in the range 0·40–0·42 had no appreciable effect on the results calculated in terms of total vitamin A alcohol and percentage of vitamin A_2 alcohol.

The results for the total vitamin A esters and alcohol content of eggs are based on four or eight individual readings, as mentioned above. In general the concentration of total vitamin A esters and alcohol was low, so that these individual readings were low, and frequently below E 0·060, when the reaction mixture had no visible blue colour. At these low extinctions, errors due to extraneous absorption may be high and such errors from the four or eight separate readings are additive. Extinctions below 0·020, corresponding to about 0·03 μg./g. wet wt., have been ignored. In addition, of the vitamin A compounds studied in this work, vitamin A_2 alcohol is the least stable. For these various reasons, the values given for concentration (or total amount) of vitamin A esters + alcohol and for the percentage of vitamin A_2 in these esters and alcohols are not as accurate as similar values for the aldehydes.

Vitamin A aldehyde in eggs

The values reported here for the three forms of vitamin A_1 and A_2 and for the carotenoids should be regarded as giving only the order of magnitude of the concentrations for the species as a whole, since in many instances only one sample was analysed, and it is well known that the vitamin A content of hen's eggs varies with the vitamin A content of the hen's diet. P. A. Plack (unpublished observations) has likewise found this variation in the vitamin A aldehyde content of hen's eggs. Presumably such a relationship between vitamin A content of the diet and vitamin A content of the egg applies to the other species, and it may explain why our first range of values for the vitamin A aldehyde content of herring ovaries (Plack *et al.* 1959; values on a wet weight basis) is higher than that found by subsequent investigators (Braekkan, Myklestad & Njaa, 1960) and higher than the value reported here.

Examined by our methods, eggs or ovaries of the five invertebrates studied contained no vitamin A, whereas those of the cephalochordate *Amphioxus lanceolatus* and ripe eggs or ovaries of all the vertebrates studied contained vitamin A aldehyde, esters and alcohol. In the two instances when unripe eggs were tested, inactive ovaries of the pike and spent ovaries of the John Dory, no aldehyde was found, thus confirming the previous finding with cod that eggs in an early stage of development do not contain vitamin A aldehyde, which appears to be laid down with the yolk (Plack *et al.* 1961). Of the vertebrates, we have analysed the eggs or ovaries from fish, an amphibian, reptiles and a bird, but not so far from the monotremes, or egg-laying mammals (e.g. duck-billed platypus and spiny anteater), or from other mammals, though collection of eggs from the monotremes is in progress.

Vitamin A has not so far been shown to be a growth factor for invertebrates, although the visual pigments of some of them do contain vitamin A aldehyde (see Fisher & Kon, 1959). In mammals, vitamin A not only takes part in the visual cycle but it is also an essential nutrient. Females deficient in vitamin A may give rise to abnormal offspring, and Hubbs & Stavenhagen (1958) have shown the same to be true of a fish, *Etheostoma lepidum* (Baird Girard). It might therefore be expected that megalecithal eggs, on which the embryos depend for some time for their food supply, should contain vitamin A, but it is not known why it should be partly in the form of vitamin A aldehyde. Winterstein & Hegedüs (1960) have recently demonstrated the presence of vitamin A aldehyde in the livers of several mammals and in some plants, and this form of vitamin A may well be of more widespread occurrence and of definite physiological importance, particularly in embryonic development.

SUMMARY

1. A method is described for the determination of vitamin A_1 and A_2 aldehydes in solutions containing mixtures of the two.

2. The eggs or ovaries of five invertebrates did not contain vitamin A aldehyde, but ovaries of a cephalochordate and ripe eggs or ovaries of two species of lamprey, four of marine elasmobranch fish, six of marine teleost fish, five of freshwater teleost, one anadromous teleost, an amphibian, five species of reptile and a bird all contained vitamin A

aldehyde. Vitamin A_1 aldehyde was the predominant form in all except the freshwater perch, pike, and trout and the anadromous salmon, which contained predominantly vitamin A_2 aldehyde.

3. Values are given for the content of total vitamin A_1 and A_2 esters and alcohols, the percentage of the A_2 form and the carotenoid content of the eggs or ovaries.

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The Assay of Insulin Solutions by Paper Chromatography

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The quantitative determination of insulin by paper chromatography has been the subject of numerous publications in recent years. Using the system butanol–acetic acid–water, in varying proportions, Light & Simpson (1956*a*, *b*) and Grodsky & Tarver (1956) separated insulin from crude pancreatic and liver proteins, Robinson & Fehr (1952) developed a method for the estimation of insulin and protamine in protamine zinc-insulin preparations, and Bouman & Homan (1958) determined insulin potency by radial chromatography. Fenton (1959) described a method for the chromatographic assay of crystalline insulin by using the solvent system butan-2-ol–1 % acetic acid (1:1, v/v). This method has since been used in these and other Laboratories, and the procedure has been extended to the estimation of insulin in solutions of composition similar to insulin injection, B.P., for which the present method describes a four-point slope ratio assay. For reasons given in the Discussion it is unlikely that these methods *in vitro* will ever be used in place of the orthodox biological assays of insulin, but attention has once again been drawn (Rückert & Schöne, 1960) to their value for production control and experimental purposes. These authors reported a simplified chromatographic procedure by which they obtained results in close agreement with those of biological assay, but they were unable quantitatively to assay residual insulin in solutions during thermal-decay tests.

EXPERIMENTAL

Materials and methods

Protein precipitant. This was a saturated aqueous solution of picric acid.

Acidified acetone reagent. Prepared by diluting 2 ml. of conc. HCl to 1 l. with acetone B.P.C. (Thos. Morson and Son Ltd.).

Developing solvent, eluent, volumetric vessels, and protein stain. These were used as described by Fenton (1959), but the following procedure must be adopted in preparing the protein stain. Bromocresol green (0·425 g.) (Hopkin and Williams Ltd.) was dissolved in 125 ml. of 0·1 N-NaOH by sprinkling the dye on the surface of the alkali and stirring. The solution was then added to 2 l. of 0·1 N-acetic acid and the mixture filtered. The reaction should be pH 3·6; the

staining solution may be used for a long time if it is filtered at intervals.

Insulin standards and samples were applied to chromatography papers with an Agla micrometer syringe (Burroughs Wellcome and Co. Ltd.), which was cleaned by washing with water, then dried with acetone when changing from one solution to the next. All precipitates were collected by centrifuging for 10–15 min. at 2000 rev./min., on an M.S.E. medium-sized centrifuge.

Standards. A stock of four-times-recrystallized zinc insulin was distributed in approx. 20 mg. amounts in hard-glass tubes and dried *in vacuo* over P_2O_5 to a moisture content of approx. 1·6%. The tubes were flame-sealed and stored at 2°. The potency of the standard was determined chromatographically (Fenton, 1959) in terms of the Fourth International Standard for insulin.

Solutions of working standard of potency approx. 40 i.u./ml. were prepared in duplicate and in sufficient quantity to last for one week's assays by dissolving accurately weighed amounts of the crystalline insulin in 0·05 N-HCl containing 0·3% *o*-cresol and 0·9% NaCl. The precise potencies were calculated to four significant figures. A four-place analytical balance and grade A volumetric glassware were used throughout; the standard solutions were stored at 2°. Subsequently it was convenient and economical to prepare standard solution in bulk, determine the potency with reference to the crystalline standard and store the solution frozen at −30° for daily use. The justification for this procedure is dealt with in the presentation of results.

Procedure

Desalting and concentration of samples. Two 5 ml. portions of standard solution (40 i.u./ml.) and two known volumes of test sample were pipetted into 100 ml. centrifuge tubes. The precise volumes of test solution were chosen from the expected potency of the sample so that approx. 150–250 units were taken. Zinc-insulin suspensions and protamine zinc insulins were dissolved by stirring rapidly with a glass rod wetted with a trace of 5 N-HCl and kept for 30 min. before sampling. Protein precipitant (2 vol.) was added with good mixing and the tubes kept for 30 min. The protein picrate precipitates were collected on the centrifuge and the clear supernatants discarded. The picrate precipitates were dissolved in 1–1·5 ml. of acidified acetone reagent, then the protein hydrochloride was reprecipitated from the solution by the rapid addition of an excess (60–70 ml.) of acidified acetone reagent.

The suspensions were stirred thoroughly with glass rods to ensure complete flocculation, covered with cellophan caps and kept for 30 min. The precipitates were collected by centrifuging, the supernatants rejected, and excess of acetone was evaporated from the precipitates by directing a gentle stream of air from a glass jet into the centrifuge tubes. The moist precipitates were dissolved in 5–10 drops of 0·05 N-HCl, and each solution was transferred to a 1 ml. volumetric vessel by means of a capillary transfer pipette. The centrifuge tubes were washed repeatedly with portions (5–10 drops) of 0·05 N-HCl, each wash was transferred to its volumetric vessel, 3–5 min. being allowed for the walls of the centrifuge tubes to drain between transfers, and the contents of all volumetric vessels were adjusted to the calibration mark and mixed thoroughly. Tests and standards were treated in the same way.

Chromatography. Four sheets of Whatman no. 1 chromatography paper were cut, folded and marked out to dimensions described by Fenton (1959) and the concentrated samples and standards applied with a micrometer syringe. In this assay 16 spots were distributed between the four papers in the following Latin-square order:

Paper 1	S1	T1	S2	T2
Paper 2	T1	S2	T2	S1
Paper 3	S2	T2	S1	T1
Paper 4	T2	S1	T1	S2

where S1 = standard at low concn. level, S2 = standard at high concn. level, T1 = test at low concn. level, and T2 = test at high concn. level. The precise volumes to be expelled from the micrometer syringe were calculated so that approximately 1 unit of insulin per spot was delivered at each low level of test and standard, and about twice this amount at each high level. Since the diameter of each spot should not exceed 1 cm. (equivalent to about 10 μl.), volumes in excess of this amount were applied in portion, each portion being allowed to dry at room temperature between applications.

For each complete assay, the four papers were placed in one tank (height 15 in., base 11 in. × 8 in.), fitted with a retractable-rod assembly as described by Fenton (1959). The chromatograms were allowed to equilibrate for not less than 16 hr. (overnight), then developed for 7½–8 hr. during the following day. Subsequent stages were carried out as described by Fenton (1959), up to calculation of results.

Calculations. The following data were collected for each complete assay: potency of standard, P (i.u./ml.); calc. vol. (μl.) of standard/spot, Z_1 (low level) and Z_2 (high level); calc. vol. (μl.) of test/spot, Z_3 (low level) and Z_4 (high level). The mean extinctions of four eluates of Z_1, Z_2, Z_3 and Z_4 are Y_1, Y_2, Y_3 and Y_4 respectively. The calculated volumes were obtained by multiplying the volume applied per spot by V_1/V_2, where V_1 is the volume of original sample or standard (ml.) and V_2 is the capacity (ml.) of the volumetric vessel.

If the results of an assay are expressed graphically (Fig. 1), the potency of the test sample can be calculated simply from the ratio of the volumes of test/standard which

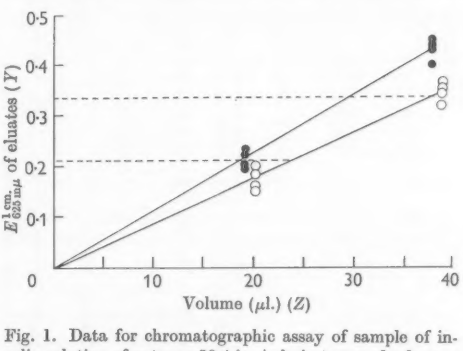

Fig. 1. Data for chromatographic assay of sample of insulin solution of potency 30·4 i.u./ml., in terms of reference standard solution (stored frozen), of potency 39·67 i.u./ml. ●, Standard, $Z = 87{\cdot}664\,Y + 0{\cdot}264$; ○, test, $Z = 112{\cdot}635\,Y + 0{\cdot}7898$; - - - - -, values of Y within range of observations. Volume was calculated in terms of original solution.

Table 1. *Data and calculation of relative potency of an insulin solution (see also Fig. 1)*

The use of a calculating machine for computing the data in this table made it convenient to retain several places of decimals throughout; values were rounded off at the end of the calculation.

Standard (frozen solution) of potency $P = 30{\cdot}67$ i.u./ml.

$V_1 = 5$ ml. $V_2 = 1{\cdot}3202$ ml. for Z_1 $V_1 = 0{\cdot}213$.
$V_2 = 1{\cdot}3233$ ml. for Z_2 $V_2 = 0{\cdot}428$.

5 μl. applied per spot for Z_1. $\therefore Z_1 = 5 \times \frac{5}{1{\cdot}3202} = 18{\cdot}9365$ μl.

10 μl. applied per spot for Z_2. $\therefore Z_2 = 10 \times \frac{5}{1{\cdot}3233} = 37{\cdot}7843$ μl.

Regression coefficient for standard line,

$$b_s = \frac{37{\cdot}7843 - 18{\cdot}9365}{0{\cdot}428 - 0{\cdot}213} = 87{\cdot}664.$$

$$C_s = 18{\cdot}9365 - \frac{0{\cdot}213\ (37{\cdot}7843 - 18{\cdot}9365)}{0{\cdot}428 - 0{\cdot}213} = 0{\cdot}264.$$

Range of observation,

$E_{625\ m\mu}$ (Y)	$(Yb_s) + C_s$	$(Yb_t) + C_t$
0·20	17·797	23·317
0·25	22·180	28·948
0·30	26·563	34·580
0·35	30·946	40·212

Test sample

$V_1 = 5$ ml. $V_3 = 1{\cdot}2539$ ml. for Z_3 $V_3 = 0{\cdot}170$.
$V_2 = 1{\cdot}2575$ ml. for Z_4 $V_4 = 0{\cdot}346$.

5 μl. applied per spot for Z_3. $\therefore Z_3 = 5 \times \frac{5}{1{\cdot}2539} = 19{\cdot}9377$ μl.

10 μl. applied per spot for Z_4. $\therefore Z_4 = 10 \times \frac{5}{1{\cdot}2575} = 39{\cdot}7614$ μl.

Regression coefficient for test line,

$$b_t = \frac{39{\cdot}7614 - 19{\cdot}9377}{0{\cdot}346 - 0{\cdot}170} = 112{\cdot}635.$$

$$C_t = 19{\cdot}9377 - \frac{0{\cdot}170\ (39{\cdot}7614 - 19{\cdot}9377)}{0{\cdot}346 - 0{\cdot}170} = 0{\cdot}7898.$$

$$\frac{[(Yb_s) + C_s].P}{(Yb_t) \times C_t} = \text{potency of sample (i.u./ml.)}$$

30·2786
30·3052
30·4729
30·5289

Geometric mean potency = 30·4 i.u./ml.

give equivalent extinctions at any value of $E_{625\,m\mu}$ within the range of observations indicated by the dotted lines. It is more precise, however, to tabulate the results as shown in Table 1, and calculate the regression coefficients for standard and test lines in terms of the linear equation $Z = bY + C$, by substitution of the data. Thus for standards:

$$b_s = \frac{Z_2 - Z_1}{Y_2 - Y_1} = \text{slope constant},$$

$$C_s = Z_1 - \frac{Y_1(Z_2 - Z_1)}{Y_2 - Y_1} = \text{(intercept)}.$$

Coefficients b_t and C_t for the test line were derived from the same equations by substituting the test data and a number of potencies for test calculated as:

$$p\ \text{(i.u./ml.)} = \frac{[(Yb_s) + C_s]\,P}{(Yb_t) + C_t},$$

taking Y at different values of extinction within the range of observations. As may be expected, the several estimates of potency were distributed more closely about their geometric mean when the differences between C_s and C_t were small. When in any assay $C_s = C_t = 0$, a single potency estimate is obtained and it is superfluous to take more than one value of Y.

Alternative methods of calculation for a four-point assay may be found in standard works; for example, the method of Finney (1952), when applied to chromatographic assays designed on the Latin square, gave satisfactory evidence of validity and enabled fiducial limits to be estimated. For this purpose the ratios of high to low level, each of test and of standard applied per spot, were kept constant at precisely 2:1, in order to fulfil the conditions required by this method of calculation.

RESULTS

The quantitative efficiency of desalting and concentration was established by assaying dilutions of insulin solution containing salts and preservative and comparing the results with those obtained by direct assay (Fenton, 1959) of comparable salt-free dilutions.

The effects of storing standard solutions in the frozen state were investigated in two separate experiments. Table 3 shows the potencies of the contents of duplicate ampoules assayed in terms of the crystalline reference standard at fixed time intervals during 8 weeks' storage at $-30°$, with one extra freezing and thawing included in the design.

When the analysis of variance on the results (Table 4) revealed no evidence suggestive of deterioration, we investigated the effects of multiple freezing and thawing in the second experiment in which insulin solution (potency 40 i.u./ml.), contained in 60 sealed glass ampoules, was frozen by immersing in a mixture of industrial methylated spirit and solid carbon dioxide, then allowed to thaw at room temperature. Groups of 20 ampoules were isolated after 5, 15 and 30 complete cycles of freezing and thawing and their contents assayed against the reference standard (crystalline insulin) as described in the experimental section. Table 5 shows the results. No evidence was found that the number of freezing cycles over the range 5–30 had any significant effect on potency. The overall variance of the 60 pooled results gave a standard

Table 2. *Recovery of insulin by chromatographic assay*

Solution *A* contained insulin and 0·05 N-HCl only, with the required amount of solution applied directly to the papers by repeated addition of 10 µl. portions; solution *B* contained insulin solution at pH 3, containing 0·9% NaCl and 0·3% *o*-cresol, chromatographed after desalting and concentration as described in the text. Theoretical potency was calculated from biological assay of the crystalline insulin from which the solutions were prepared.

Theoretical potency (i.u./ml.)	Potency by chromatographic assay (i.u./ml.)	
	A	*B*
42·0	42·0	43·6
36·5	36·9	36·6
32·0	31·5	32·0
25·5	26·0	24·8
21·0	21·0	22·4
12·5	10·5	11·1

Table 3. *Potency of insulin solutions assayed during storage at* $-30°$

Compositions of solution: insulin 40 i.u./ml.; NaCl, 0·9%; *o*-cresol, 0·3%; dil. HCl to pH 3. Solutions were stored in 10 ml. sealed glass ampoules.

Period of storage at $-30°$ (weeks)	Potency of solution (i.u./ml.)			
	Single freezing and thawing (duplicate ampoules)		Double freezing and thawing (duplicate ampoules)	
0	40·0	—	40·0	—
1	41·5	38·5	39·9	—
2	39·8	41·8	40·9	40·0
3	40·0	40·1	39·7	39·8
5	42·4	42·2	42·5	41·8
8	41·2	40·0	40·6	40·4

Table 4. *Analysis of variance for data in Table* 3 *after transformation to log potencies* (*abridged table*)

Source of variance	Degrees of freedom	Mean square
Between times of testing	5	0·00029628
Between frequencies of freezing	1	0·00008932
Interaction, times and frequency	5	0·00000275
Between duplicates	11	0·00009367

deviation of ±4·5% per assay, compared with ±4·3% associated with the estimation of crystalline insulin reported by Fenton (1959).

The results of the chromatographic determination of 27 samples of insulin solution of composition corresponding to insulin injection, B.P., are summarized in Fig. 2. Statistical analysis of the results gave a very significant correlation (r 0·98, D.F. 25, $P < 0·001$) between the results and those of biological assay on the same samples.

Table 5. *Effect of successive cycles of freezing and thawing on insulin solutions*

Composition of solutions: insulin, 40 i.u./ml.; NaCl, 0·9%; *o*-cresol, 0·3%; dil. HCl to pH 3. Solutions were stored in 10 ml. sealed glass ampoules.

No. of cycles of freezing and thawing	Potency (mean of 20 ampoules) (i.u./ml.)	Limits of error, P 0·05 (i.u./ml.)
0	40·0	39·4–40·6
5	40·0	39·4–40·6
15	39·6	39·1–40·9
30	39·4	39·1–40·9

DISCUSSION

Comparisons between results obtained by chromatographic and biological assays on the same solutions have shown that the best agreement is obtained when, in the chromatographic determination, the amount of insulin present in the developed spots is the same for test and standard. This observation agrees with that of B. G. Overell (personal communication). The present design enables equivalent amounts of test and standard to be compared each at two working levels and fulfils the requirements of a slope ratio assay of the type in which two lines of different slope, intersecting at or close to the blank level, represent the conventional dose–response relationship. If b_s and b_t are estimates of the two regression coefficients, $R = (b_s/b_t)$ is an estimate of relative potency.

The response parameter is a spectrophotometric determination of eluate intensity, but the customary practice of referring experimental points to a fixed standard curve is not permissible because of the inherent errors introduced by positional effects of spots within papers, errors between papers

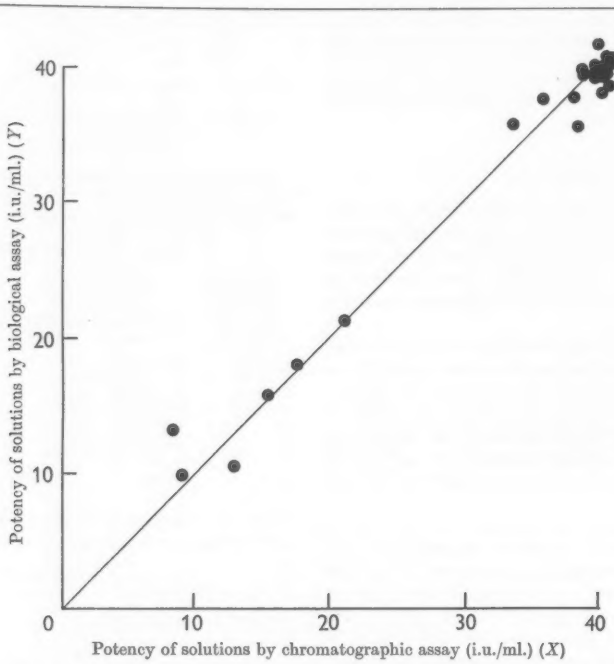

Fig. 2. Results of the chromatographic assay of 27 samples of insulin solutions compared with the results of biological assay. The regression line is the diagonal, $X = Y$. The co-ordinate values, after transformation to logarithms to base 10, gave $X = 1·08\,Y - 0·12$ and $Y = 0·885X - 0·173$ for the best-fitting lines.

(Fenton, 1959) and temperature fluctuations (Bouman & Homan, 1958). These effects had to be compensated by the presence of standards on every paper in each assay. The Latin-square design balanced out positional and paper effects, since each spot of test and standard occurred once on each paper and occupied all available positions over the four papers.

Several unsuccessful attempts were made to separate insulin from crystalline glucagon (Staub, Sinn & Behrens, 1953, 1955), by the chromatographic method as described and by the radial technique of Bouman & Homan (1958). Although the latter procedure gave somewhat better separation in our hands than the ascending method, there was still some overlapping of the spots. We regard glucagon therefore as a possible source of interference. It has been our experience that on occasions when chromatographic and biological assays disagree on identical samples the higher value is usually the chromatographic one. Although it would be illogical to ascribe this to the specific effect of glucagon, the phenomenon is consistent with our finding that the two substances migrate to almost the same R_F position in the butan-2-ol–acetic acid–water system. There may be other substances of pancreatic origin which behave in a similar way. These findings do not necessarily detract from the value of chromatographic methods for the estimation of insulin but point to the clear distinction to be made between the estimation of insulin in samples of known history and its detection in the presence of foreign substances.

SUMMARY

1. A method for the assay of insulin solutions by ascending paper chromatography is described. The solutions, after desalting and concentration, are applied to papers which are developed in the upper phase of butan-2-ol–1% acetic acid (1:1, v/v), then stained with bromocresol green. The dye is eluted and its concentration measured in a spectrophotometer with respect to standards run simultaneously. Treatment of the data as a four-point slope ratio assay gave results in good agreement with those obtained on the same solutions by the mouse convulsion method.

2. Solutions of four-times-recrystallized insulin, stored in the frozen state for use as working standards, remained stable for periods up to 8 weeks at −30°, and could be refrozen and thawed up to 30 times in the presence of phenolic preservative without detectable loss of potency.

I am indebted to Dr D. W. Adamson (Research Director, The Wellcome Foundation Ltd.) and to the Directors of the British Insulin Manufacturers member companies for permission to publish. I also wish to thank Dr D. R. Bangham (Department of Biological Standards, National Institute for Medical Research, London, N.W. 7) for his keen interest in the progress and results of the work on storage of frozen sub-standards and for supplies of the 4th International standard for insulin; Dr C. G. Pope, under whose guidance the work was carried out; Dr G. A. Stewart, for biological assays; and Mr P. A. Young and Mr G. J. Knight for statistical analyses. The valuable technical assistance of Miss V. Putley, Miss A. Rogers and Miss S. Brooks is also gratefully acknowledged.

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Inhibition of the Biosynthesis of Cell-Wall Mucopeptides by the Penicillins

A STUDY OF PENICILLIN-SENSITIVE AND PENICILLIN-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

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Previous work has shown that benzylpenicillin can inhibit cell-wall-mucopeptide synthesis by staphylococci (Park, 1958; Mandelstam & Rogers, 1959). The precise relationship, however, between the concentrations of the antibiotic required to do this and those necessary to prevent growth of the organisms has not been examined in detail. In the work described by Mandelstam & Rogers (1959), a 'naturally' resistant strain of the organism was used (i.e. strain 524/SC). It was originally isolated from a human patient before re-isolation by single-cell selection (Rogers, 1953). Such 'naturally resistant' strains produce inducible penicillinase, and although the organism was grown in the absence of penicillin and examined in the presence of chloramphenicol in this earlier work (Mandelstam & Rogers, 1959), residual penicillinase was likely to have been present in the suspensions. As a consequence no attempt was made to examine the precise concentration of benzylpenicillin required to inhibit mucopeptide synthesis. Staphylococci selected in the laboratory from media containing penicillin, however, do not produce detectable amounts of penicillinase (Barber, 1953). If the antibiotic action of benzylpenicillin on both sensitive and resistant strains is due to inhibition of mucopeptide synthesis, then one would expect the lethal concentrations to coincide with those required to cause significant inhibition of mucopeptide synthesis.

Two new penicillins derived from 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959) with different side chains have now been described (Stewart, Harrison & Holt, 1960; Rolinson, Stevens, Batchelor, Wood & Chain, 1960). Again, if the antibiotic action of these compounds is due to their ability to inhibit mucopeptide synthesis, the lethal concentrations and those required to inhibit mucopeptide synthesis should bear a constant relationship. The effects upon mucopeptide synthesis of various concentrations of the new penicillins and of benzylpenicillin have now been examined, with the sensitive *Staphylococcus aureus* strain Oxford, and a resistant substrain derived from it. The results obtained provide strong evidence in favour of the hypothesis, first put forward by Park & Strominger (1957), that the primary action of penicillin is upon the formation of cell wall. Although the position with Gram-negative forms may be more complicated, it seems probable that the hypothesis is correct for the Gram-positive staphylococci. The effect of one of the new penicillins, sodium 6-(2:6-dimethoxybenzamido)penicillinate (AB 1241, Methicillin, Celbenin), upon mucopeptide synthesis by the penicillinase-producing *S. aureus* strain 524/SC has also been examined. If the presence of penicillinase in the cells influences the inhibition produced by benzylpenicillin (Mandelstam & Rogers, 1959), 6-(2:6-dimethoxybenzamido)penicillin should be relatively more effective against this strain than the benzyl derivative. This penicillin is not readily hydrolysed by staphylococcal penicillinase (Rolinson *et al.* 1960; Knox, 1960).

METHODS AND MATERIALS

Strains of organism. *S. aureus* strain Oxford in a dried form was obtained from Mr J. Lightbown of this Institute. Strain 524/SC was as used previously (Mandelstam & Rogers, 1959).

Testing of penicillin sensitivity and selection of penicillin-resistant substrain. A subculture of *S. aureus* Oxford in broth (see below) was tested for sensitivity to penicillin by inoculating (*a*) a series of flasks of Hedley–Wright broth (Rogers, 1953) containing increasing amounts of penicillin; (*b*) a series of Petri dishes containing the same medium solidified with 2% of agar and containing increasing concentrations of benzylpenicillin. Growth was found to be inhibited by 0·036 μg. of benzylpenicillin/ml. but not by 0·024 μg./ml. on agar plates, and by 0·12 μg./ml. but not by 0·06 μg./ml. when growing in broth. In order to select pencillin-resistant substrains, two techniques were used: (*a*) the gradient plate technique of Szybalski & Bryerson (1952) and (*b*) the selection of colonies from heavily inoculated agar plates containing sufficient penicillin almost to inhibit growth completely. As an example, a plate containing, say, 0·036 μg. of penicillin/ml. was inoculated with the original sensitive strain and a few colonies appeared

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(growth up to 76 hr. was necessary at some stages of the selection). One of these was selected and a culture grown from it in liquid medium containing 0·036 μg. of penicillin/ml. This culture was subcultured on a plate containing a slightly higher concentration of the antibiotic, and so on. By these processes, substrains resistant to concentrations of penicillin of 2·1 μg./ml. up to 30 μg./ml. were obtained. For the present work only the one resistant to 2·1 μg./ml. (i.e. growing in 2·1 μg./ml. but not in 2·4 μg./ml.) was used. This strain very readily reverted to penicillin sensitivity, and growth through two or three subcultures in the absence of antibiotic considerably decreased its resistance. During the course of the work the resistance of the strain declined. The strain was then reselected from agar plates containing 1·8 μg. of penicillin/ml. When the reselected strain was tested it was found to grow in 24 hr. broth containing 9·5 μg. of penicillin/ml., but not 19·0 μg./ml. The first resistant substrain isolated will be distinguished as no. 1 from the re-isolated substrain, to be called no. 2.

Penicillin. Commercial benzylpenicillin (Glaxo Laboratories Ltd.) in phials containing 10^5 i.u. (i.e. 60 mg.) was used. For critical experiments the precise strength of solutions made from this material was tested by comparison with solutions of the British Standard Penicillin, having a potency of 1·680 i.u./μg. The latter was given to us by Mr J. Lightbown of this Institute. 6-(α-Phenoxypropionamido)penicillanic acid (Broxil), 6-(2:6-dimethoxybenzamido)penicillin (AB 1241, Methicillin, Celbenin) and 6-aminopenicillanic acid were obtained through the courtesy of Dr M. R. Pollock and Dr D. L. Swallow.

Broth medium. This was Hedley–Wright broth, containing 2·5% of sodium β-glycerophosphate as previously described (Rogers, 1953). This medium was substituted for the casein-hydrolysate growth medium previously used, because growth of the resistant substrains in the casein hydrolysate was very slow and did not occur at all when benzylpenicillin at the concentration at which the substrain had been selected was included to preclude back-mutation to sensitivity. It was subsequently shown that back-mutation did not occur in sufficient cells during the short growth period used to influence the results, and the antibiotic was therefore omitted from the broth medium. Even in broth medium and in the absence of the antibiotic, growth of the resistant substrains had a considerable lag period and frequently stopped much sooner than that of the normal strain. The technique for growing the resistant strains was standardized as follows. Cultures of the organism grown on agar containing 1·8 μg. of benzylpenicillin/ml. for 24 hr. were dried by the Stamp (1947) technique. A new dried culture was then used for each experiment. It was inoculated into 10 ml. of Hedley–Wright broth medium containing 1·8 μg. of benzylpenicillin/ml., and incubated with shaking at 35° for 18 hr. This culture (5·0 ml.) was then added to 200 ml. of the same medium without penicillin and incubated under the same conditions for 7 hr., cooled and stored at 0–4° overnight. The next morning it was diluted with an equal volume of broth and incubated until the extinction of the culture had doubled. After this the suspension was prepared as previously described.

Conditions for cell-wall biosynthesis. Suspensions were prepared and incubated as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960); all four of the mucopeptide amino acids and chloramphenicol (50 μg./ml.) were always present unless otherwise stated. Penicillin solutions, when required, were added along with the other ingredients before addition of the cell suspension. Incubation was for 90 min., unless otherwise stated, at 35°, and the flasks were shaken in order to aerate the suspensions.

Preparation of mixed trichloroacetic acid precipitates and cell walls. These were prepared as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960).

Analytical methods, including radioactivity determinations. These were as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960); 1000 counts above background were always collected. It was, however, not found possible to measure cell-wall synthesis by the increase in extinction of the suspensions when strain Oxford was used.

Radioactive amino acids. [1-^{14}C]Glycine, [1-^{14}C]alanine, [1-^{14}C]glutamic acid and generally ^{14}C-labelled lysine were obtained from The Radiochemical Centre, Amersham, and initially diluted with unlabelled amino acids so that 1 mg. ≡ 1 μc; these materials were then diluted a further 10-fold with the corresponding unlabelled compound in the test system.

RESULTS

Effect of age of the culture

In all the previous work (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960), the cells used for preparing suspensions to study cell-wall synthesis were obtained from rapidly growing cultures in casein-hydrolysate media. Both *S. aureus* strain 524/SC and the original Oxford strain grew rapidly in casein-hydrolysate media, thus allowing completion of the experiment in a reasonable time. The selected benzylpenicillin-resistant substrains, however (see Methods and Materials section), grew very slowly and often to a very low final culture density in casein hydrolysate. At one stage in the work it was thought that in order to obtain enough organisms and to perform the experiments in a reasonable period of time, it might be possible to use cultures incubated overnight, i.e. cultures in which cells had ceased to multiply rapidly. To test this possibility a comparison was made between suspensions prepared from cultures of the normal Oxford strain in which the organisms were growing rapidly (Mandelstam & Rogers, 1958) and from cultures in which the cells had ceased to multiply. Broth medium (cf. Methods and Materials section) was used. Suspensions were prepared in the usual way and their ability to incorporate [1-^{14}C]glycine was measured in the presence and absence of two concentrations of penicillin (Table 1). The cells from overnight cultures differed from those obtained by the standard procedure, which employed cells that had been growing rapidly, in two important ways. The total incorporation of the amino acid in the period of incubation and hence mucopeptide synthesis was less, and the higher concentrations of benzylpenicillin were less inhibitory to

Table 1. *Effect of benzylpenicillin upon staphylococci from cultures of different ages*

Strain: *Staphylococcus aureus* Oxford, normal. A, Strain incubated for 24 hr. at 37° in Hedley–Wright broth supplemented with glucose and sodium β-glycerophosphate. B, Strain incubated for about 4 hr. to the density of 1 mg./ml.; it was left overnight in the cold, warmed, the same volume of medium was added and incubated to density 1 mg./ml. The suspensions of washed organisms were adjusted to the density of 1 mg./ml. to study incorporation of [1-^{14}C]glycine. The radioactivity at infinite thickness of hot trichloroacetic acid precipitates is given.

Time of incubation: 90 min.

Benzylpenicillin (μg./ml.)	Counts/min.		Inhibition (%)	
	A	B	A	B
0·012	68·4	143·1	15·6	1·6
0·024	65·5	130·0	19·2	10·6
0·048	54·5	90·0	32·8	38·1
0·060	50·6	77·8	37·6	46·5
0·30	47·7	54·8	41·2	62·3
0·60	43·7	55·5	46·1	61·8
1·2	40·6	44·4	49·9	69·5
0	81·0	145·2	—	—

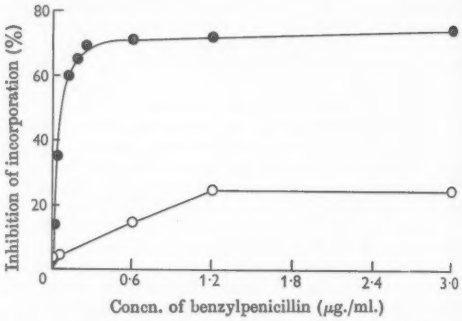

Fig. 1. Inhibition of the incorporation of [1-^{14}C]glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of the sensitive (●) and resistant (no. 2) substrain (○) of *S. aureus* strain Oxford. The suspensions had been incubated under the conditions set out in the Methods and Materials section. In the absence of penicillin the radioactivity of the preparations from both strains was equivalent to about 80 counts/min.

the process. Since one of the purposes of the present work was to compare the effects of benzylpenicillin upon the sensitive and resistant substrains, all further work was done with rapidly growing cells (i.e. cells treated by the standard procedure). This experiment stresses the need for careful control of the growth conditions for the micro-organisms in studies of mucopeptide biosynthesis.

Effect of benzylpenicillin on incorporation of cell-wall amino acids

The cell suspensions of both strains were prepared in the usual way from cultures of exactly the same age. Both suspensions in phosphate–glucose solution were added to the usual mixture of the four mucopeptide amino acids, penicillin and chloramphenicol. In one experiment the glycine was labelled, and in the other glutamic acid was labelled. Concentrations of benzylpenicillin including those to which the cells were sensitive when growing in liquid and solid media (i.e. about 0·03–0·06 μg./ml. for the sensitive strains and 2·4–12·0 μg./ml. for the resistant strain) were employed. After incubation mixed hot trichloroacetic acid precipitates were prepared and their radioactivity was measured at infinite thickness. Resistant substrain no. 1 was used.

The incorporation by the sensitive strain of labelled glutamic acid is significantly inhibited by doses of benzylpenicillin in the range 0·018–0·06 μg./ml. (Fig. 1). The difference between duplicate tests within a single experiment has been found to be about 10% over a large number of experiments. Similar results were obtained for the incorporation of glycine.

Comparison of the behaviour of the resistant and sensitive substrains (Fig. 1) clearly shows the difference between them. At a concentration of 0·06 μg./ml. or more of benzylpenicillin the two strains differ markedly for the incorporation of [1-^{14}C]glutamic acid. At a concentration of 0·6 μg./ml. of antibiotic only the resistant strain grew. Even when the concentration of benzylpenicillin was increased to 6·0 μg./ml., glutamic acid incorporation by the resistant strain was inhibited by only 40% and glycine incorporation by 60%. At 24·0 μg./ml. glycine incorporation was decreased by 70%, an inhibition still somewhat less than that caused by 1·2 μg./ml. with the sensitive strain.

Table 2 shows the effect of three concentrations of benzylpenicillin on the incorporation by the sensitive substrain of each of the four amino acids present in the mucopeptide; at a concentration equivalent to 0·03 μg./ml., inhibition of incorporation of all four amino acids was between 23 and 36%.

When the concentration of antibiotic was increased 100-fold, to 3·0 μg./ml., the incorporation of the amino acids by the sensitive strain was not completely stopped. In the earlier experiments this seemed to apply particularly to the incorporation of [1-^{14}C]alanine. When these experiments were repeated, however, some months later, rather higher degrees of inhibition were obtained, as for example in Expts. 3 and 4 of Table 2, and the difference between the behaviour with this amino acid and the others was then less marked. The incomplete

Table 2. *Comparative inhibition of the incorporation of the four cell-wall amino acids into cell-wall mucopeptide by benzylpenicillin acting on the sensitive substrain*

The techniques were as given in the Methods and Materials section. The radioactivity of hot trichloroacetic acid precipitates was measured.

Concn. of penicillin (μg./ml.)	Expt. no.	Alanine		Glutamic acid		Glycine		Lysine	
		Counts/ min.	Inhibition (%)	Counts/ min.	Inhibition (%)	Counts/ min.	Inhibition (%)	Counts/ min.	Inhibition (%)
0	1	148	—	72	—	98	—	119	—
0·03	1	114	23	46	36	73	25	86	28
1·2	1	62	58	20	72	25	74	52	56
3·0	1	66	53	19	74	—	—	49	59
0	2	170	—	—	—	106	—	—	—
3·0	2	61	64	—	—	20	81	—	—
0	3	150	—	—	—	—	—	—	—
3·0	3	36	76	—	—	—	—	—	—
0	3	258*	—	—	—	—	—	—	—
3·0	3	76*	71	—	—	—	—	—	—

* Approximately twice the usual concentration of [1-^{14}C]alanine was used in this experiment.

inhibition with higher doses of antibiotic is largely due to a short initial period of rapid incorporation which is dealt with more fully in the next section of this paper.

Time-course of incorporation of amino acids by the sensitive strain in the presence of penicillin

In these experiments separate flasks containing a suspension of the sensitive cells in a solution of the usual four amino acids (one of which was labelled), chloramphenicol, glucose and phosphate, with or without benzylpenicillin, were incubated at 35° with shaking. Pairs of flasks were removed at the times indicated by the points on Fig. 2, which illustrates the results obtained for glutamic acid incorporation. Most of the incorporation which took place in the presence of the higher concentrations of antibiotic occurred during the first 10–20 min., and there was only a very slow continuous deposition after this. If this period of rapid incorporation is ignored, the subsequent inhibitions of mucopeptide synthesis deduced from Fig. 2 are 47, 72 and 96 % respectively for concentrations of 0·06, 0·12 and 12·0 μg. of benzylpenicillin/ml.

Comparison of mixed trichloroacetic acid precipitates with cell-wall preparations

Eight separate flasks containing the usual components for studying mucopeptide synthesis were set up: four contained 1·2 μg. of benzylpenicillin/ml. and four served as controls. A different amino acid was labelled in each of the four pairs. Thus a comparison could be made of the effect of 1·2 μg. of benzylpenicillin/ml. upon the incorporation of each of the four mucopeotide amino acids. The sensitive strain of *S. aureus* Oxford was used. At the end of the usual 90 min. incubation, the suspensions were cooled, a sample was withdrawn for the preparation of mixed trichloroacetic acid precipitates and the remainder was centrifuged, the cells were washed and the cell-wall preparations made as before (Mandelstam & Rogers, 1959). The radioactivity in all the preparations was measured at infinite thickness.

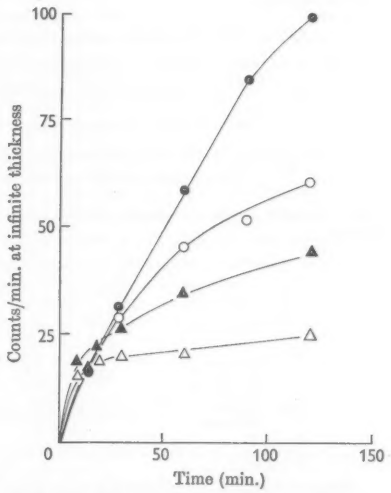

Fig. 2. Influence of various concentrations of benzylpenicillin upon incorporation of [1-^{14}C]glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of the sensitive strain of *S. aureus* Oxford. The suspensions were incubated under the usual conditions for cell-wall-mucopeptide synthesis. The flasks contained: ●, no penicillin, this served as the control; ○, 0·12 μg. of benzylpenicillin/ml.; ▲, 1·2 μg. of benzylpenicillin/ml.; △, 12 μg. of benzylpenicillin/ml.

As can be seen from Table 3, in most instances the agreement between the degree of inhibition of incorporation into the cell-wall preparations and into the material insoluble in hot trichloroacetic acid, which also includes the proteins of the cells, was within experimental error. Thus it seems justified to conclude, as might be expected on the basis of earlier work (Mandelstam & Rogers, 1958, 1959), that results obtained with the mixed trichloroacetic acid precipitates are a good indication of incorporation into mucopeptide and hence of the cell-wall synthesis. However, some caution may still be necessary, because in two experiments with the higher concentration of antibiotic of 3·0 μg./ml. it was found that the incorporation of [1-^{14}C]-alanine into cell-wall preparations was inhibited to a significantly greater extent than into material insoluble in hot trichloroacetic acid (see Table 3, Expts. 2 and 3). The reasons for this are at present under investigation.

Composition of the cell wall of the resistant substrain

Cell walls were prepared from the sensitive and resistant strains by the method of Cummins & Harris (1956) and analysed by the usual methods (Table 4). The differences between the results for the two strains are chiefly a higher content of glutamic acid and a lower value for muramic acid in the batches of wall from the sensitive organism. However, over a period of years, variation of the analytical values obtained for different batches of wall preparations from the sensitive strain have been greater than the differences shown in Table 4 between the sensitive and resistant forms. For example, the molecular proportions for the amino acids previously quoted by Rogers & Perkins (1959) for *S. aureus* strain Oxford show such a greater difference from the present results for the same strain (sensitive strain in Table 4). Similar variation has previously been reported for *S. aureus* strain 524/SC (Mandelstam & Rogers, 1959). It is therefore doubted whether the walls from the sensitive and resistant strains can be said to differ significantly in composition. The total sum of the amino acids and amino sugars, allowing for the water of hydrolysis, accounts for about 80–85 % of the dry weight of the preparations from both strains.

Table 3. *Effect of penicillin on the incorporation of radioactively labelled amino acids into cell-wall preparations and into material insoluble in hot trichloroacetic acid*

The sensitive strain of *S. aureus* Oxford was used. Methods were as described in the Methods and Materials section.

Amino acid	Expt. no.	Dose of benzylpenicillin (μg./ml.)	Inhibition (%)	
			Cell-wall preparation	Hot acid precipitate
Alanine	1	1·2	48	47
Glutamic acid	1		63	69·7
Glycine	1		89	77
Lysine	1		42	45
Alanine	2	3·0	91	76
Alanine	3		85	71

Table 4. *Analyses of cell-wall preparations from the resistant (no. 2) and sensitive strains of* Staphylococcus aureus *Oxford*

The analytical methods are as stated in the Methods and Materials section, and the molar proportions of the components are given in relation to glutamic acid taken as 1·00. The two sets of figures given for the sensitive strain are on cell walls prepared from different batches of cells.

	Sensitive strain		Resistant strain (no. 2)	
	%	mol.prop.	%	mol.prop.
Alanine	10·2, 13·2	1·4, 1·71	11·2	1·94
Lysine	10·4, 9·4	0·87, 0·71	9·9	1·04
Glutamic acid	11·8, 12·8	1·00, 1·00	9·5	1·00
Glycine	15·4, 20·2	2·50, 3·10	16·8	3·43
Glucosamine	17·2, 15·2	1·19, 0·99	14·8	1·27
N-Acetylglucosamine	21·2, 18·8	—	18·3	—
Muramic acid	14·4, 14·8	0·70, 0·69	17·4	1·06
N-Acetylmuramic acid	17·2, 17·4	—	20·4	—
Total	86·2, 91·6	—	86·1	—
Total – water of hydrolysis	81·3, 84·7	—	80·0	—

Inhibition by the 'new penicillins' upon normal and resistant substrains of strain Oxford

The effects of Methicillin, Broxil and 6-aminopenicillanic acid upon mucopeptide synthesis were examined by the same techniques as those already described, the sensitive and resistant substrains being used (Table 5). With the sensitive strain, the ratios of the concentrations of the new penicillins to that of benzylpenicillin required to give 60–70% inhibition of mucopeptide synthesis are: Methicillin about 60–100, Broxil about 1 and 6-aminopenicillanic acid about 1000. The corresponding ratios for the concentrations required for prevention of growth are quoted by Garrod (1960) as about 60 and 1, for Celbenin and Broxil, whereas the corresponding ratio for 6-aminopenicillanic acid would appear to be about 1–2000 (unpublished observation). Thus there is rather close similarity between the relative effects of the four antibiotics on mucopeptide biosynthesis and growth. Considering now the resistant substrain, the ratios, in the same order as given above, are less than 6, rather less than 1 and greater than 100. Methicillin is very much more effective against the benzylpenicillin-resistant strain than would be expected, with the result that the ratio of the amount of Methicillin to that of benzylpenicillin required to inhibit mucopeptide synthesis is less than with the sensitive strain. It appears that organisms which are resistant to benzylpenicillin are not resistant to Methicillin, as far as mucopeptide synthesis is concerned.

Effect of Methicillin upon protein and nucleic acid formation by the sensitive Staphylococcus aureus *Oxford*

In view of the failure of cells which are resistant to benzylpenicillin to be resistant to Methicillin, it seemed necessary to check that the new antibiotic did not have some more widespread attack on the cell than inhibition of mucopeptide synthesis. To test this, two series of experiments were done to examine the effect of the antibiotic on protein and nucleic acid synthesis.

Protein synthesis. Cells were incubated in the usual system used for studying mucopeptide synthesis with the addition of 0·2 mg. of L-[^{14}C]phenylalanine (containing 0·05 μc of radioactivity)/ml. and 0·2 mg. of acid-hydrolysed casein (supplemented by tryptophan; Rogers, 1954)/ml.; chloramphenicol was omitted from all except one flask. This flask served to show that the incorporation measured in the others was in fact due to protein synthesis. The radioactivity of the material insoluble in 5% trichloroacetic acid at 90° was measured at infinite thickness.

Nucleic acid synthesis. Cells were incubated in the usual system used for studying mucopeptide synthesis. After incubation for varying periods they were washed twice with cold 5% trichloroacetic acid, the washings were discarded and the deposit was extracted at 90° for 15 min. with 5% trichloroacetic acid. The trichloroacetic acid was removed from the supernatants by extraction four times with equal volumes of ether, and the ether

Table 5. *Effect of the new penicillins upon cell-wall biosynthesis by the sensitive and resistant substrains of* Staphylococcus aureus *Oxford*

The resistant substrain was no. 2. Conditions for studying [1-^{14}C]glutamic acid incorporation were as described in the Methods and Materials section.

	Concn. (μg./ml.)	Inhibition (%) Sensitive strain	Inhibition (%) Resistant strain	Lethal dose* for sensitive strain (μg./ml.)
Benzylpenicillin	0·3	70	—	0·02–0·032
	3·0	—	35	
Methicillin	1·0	8	—	1–2·0
	2·0	19	—	
	5·0	51	—	
	10	51	46	
	20	59	69	
	50	71	—	
	100	71	85	
Broxil	0·16	51	—	0·01–0·032
	0·32	61	—	
6-Aminopenicillanic acid	0·2	3	—	~ 60
	20	28	—	
	200	—	38	
	300	72	—	
	400	—	39	

* Results taken from Garrod (1960), Knox (1960) and Rolinson, Stevens, Batchelor, Wood & Chain (1960).

removed by a stream of air. Finally the extracts were made up to 10 ml. and the extinction at 260 mμ of suitable dilutions was measured. In all the experiments, flasks which contained 60 μg. of benzylpenicillin/ml. and 100 μg. of Methicillin/ml. were compared with control flasks which contained no antibiotic.

The results were entirely negative in that, at these concentrations, neither antibiotic had any influence either upon the incorporation of [^{14}C]-phenylalanine or upon the formation of nucleic acids. The incorporation of [^{14}C]phenylalanine into the material insoluble in hot trichloroacetic acid was inhibited 96% by 50 μg. of chloramphenicol/ml. Chloramphenicol has no effect upon mucopeptide synthesis (Mandelstam & Rogers, 1958; Hancock & Park, 1958).

Effects of benzylpenicillin and Methicillin upon cell-wall formation by strain 524/SC

The organisms were grown and incorporation of [1-^{14}C]glutamic acid into the material insoluble in hot trichloroacetic acid in the presence of the antibiotics was studied as previously described (Mandelstam & Rogers, 1958, 1959). Fig. 3 shows the results obtained. It will be noted that, as was found before (Mandelstam & Rogers, 1959), inhibition of cell-wall formation by benzylpenicillin is far from complete, and even when the concentration of the antibiotic is increased very much, inhibition is not greatly increased. With Methicillin, however, inhibition up to 80–90% occurs at concentrations of Methicillin similar to those required for this effect upon the sensitive Oxford strain.

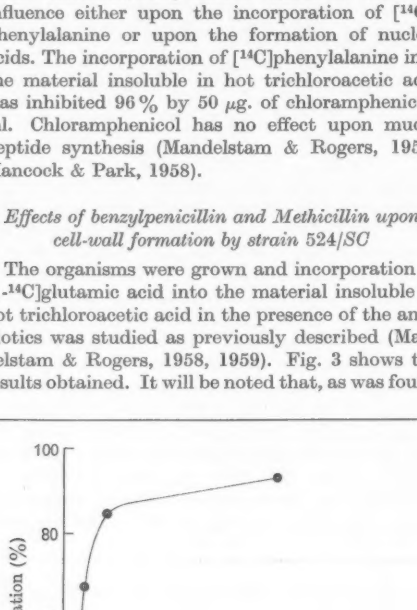

Fig. 3. Effect of different concentrations of benzylpenicillin (○) and Methicillin (●) on the incorporation of [1-^{14}C]-glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of *S. aureus* strain 524/SC after incubation under the usual conditions for the formation of cell-wall mucopeptides.

DISCUSSION

The results described in this paper are all consistent with the hypothesis originally put forward by Park & Strominger (1957), that the primary site of action of benzylpenicillin is the mechanism which forms the cell walls in staphylococci. When penicillin-resistant cells are selected on artificial media, the mucopeptide-forming system in such organisms is strikingly less sensitive to benzylpenicillin than that in cells from the original culture. The reason for the small amount of mucopeptide formation by the sensitive strain which still occurs even in the presence of relatively high doses of the antibiotic (i.e. inhibition after 90 min. incubation of the system never appears to be more than about 70–80% complete) is made clear by an examination of the time-course of incorporation. During the first 10–15 min. of incubation, penicillin has relatively little effect upon the synthesizing system; thereafter with concentrations of benzylpenicillin of 0·6 μg./ml. and more, inhibition is almost complete. Although the conditions for the assay of the effects of antibiotics upon growth of the organisms and of those for the present tests for mucopeptide synthesis are so very different, the concentrations of benzylpenicillin required to affect both are of the same order for both sensitive and laboratory-selected resistant strains. Such a comparison becomes more impressive when penicillins with different side chains are compared. The relative effectiveness of the substances for the two manifestations of the antibiotics are remarkably similar. The one apparent anomaly is that, with the cells made resistant to benzylpenicillin, mucopeptide synthesis is still sensitive to Methicillin. Such a lack of correlation is to be expected with strain 524/SC, since resistance of this latter strain is due to the production of penicillinase, and Methicillin is much less readily hydrolysed by penicillinase. With strains made resistant in the laboratory by selection, this explanation should not hold, since their resistance is not to be explained by the production of penicillinase. However, Barber (1961) found that strains selected to grow in the presence of benzylpenicillin show little or no ability to grow in higher concentrations of Methicillin. Hence even this apparent contradiction is not a real one as far as the relative effects of Methicillin on growth and

cell-wall-mucopeptide formation are concerned. Within the limitations of the examination made, the mechanism of action of Methicillin appears to be the same as that of benzylpenicillin. Of three major biosynthetic processes in the cell (namely those for proteins, nucleic acids and cell-wall mucopeptides), only the formation of the mucopeptides is affected by high concentrations of the antibiotic. This conclusion is similar to that drawn by Gooder & Maxted (1961) for streptococci.

In earlier work (Mandelstam & Rogers, 1959) it was reasoned that because the strain 524/SC had been grown in the absence of benzylpenicillin, the cells then washed well and incubated in the presence of chloramphenicol so that no more enzyme could be formed, penicillinase was not likely to play any part in the effect of the antibiotic on mucopeptide synthesis. Nevertheless it was found that mucopeptide formation continued, although at a reduced rate, for about 1 hr. in the presence of very high concentrations of penicillin before ceasing altogether. It was then argued that this must be due to the presence of a penicillin-resistant pathway for synthesis in the organisms. Moreover, the mucopeptide deposited in the presence of penicillin was different from that formed in the absence of the antibiotic. It has now been shown that when Methicillin is used, which is hydrolysed much less easily by penicillinase than is benzylpenicillin, inhibition of mucopeptide formation reaches values of 80–90% with concentrations of the antibiotic similar to those required for inhibition of sensitive strains. Hence it is possible that the previous results were due to the presence of penicillinase in the cells. However, in view of the results for the action of Methicillin upon laboratory-selected resistant mutants of strain Oxford, caution in the total rejection of the previous idea should be exercised. The behaviour of these resistant mutants is similar to that of 524/SC. Both strains require high concentrations of benzylpenicillin to effect inhibition of mucopeptide synthesis, but both are sensitive to Methicillin, yet the selected mutants of strain Oxford do not produce penicillinase, whereas, of course, 524/SC does. Hence the difference between the effects of benzylpenicillin and Methicillin upon 524/SC need not be necessarily related to the presence of penicillinase.

The hypothesis that the primary site of the lethal action of penicillin is the system responsible for mucopeptide biosynthesis has been challenged by the experiments of Hugo & Russell (1960*a*, *b*) and by those of Meadow (1960). The observations of the former authors are: (*a*) that 6-aminopenicillanic acid is much more effective in causing spheroplast formation than in killing a variety of species of Gram-negative organisms, and (*b*) that in the presence of large amounts of benzylpenicillin acting on *Escherichia coli*, the sum of the number of spheroplasts and the number of viable organisms is only about 40% of the original total number of viable organisms before the addition of the antibiotics, and it appeared unlikely that this loss was wholly due to lysis of spheroplasts. No complete answer is, of course, available to enable this work to be understood in the light of the results obtained in the present paper. However, the concentrations of benzylpenicillin used by Hugo & Russell (1960*a*, *b*) are of the order of 10^3–10^4 times those required to inhibit mucopeptide formation by the penicillin-sensitive *S. aureus*. It may be asked whether at such high concentrations the antibiotic might not have more widespread side effects on the cells than it does at the concentrations required to kill sensitive organisms. Also, the formation of spheroplasts is necessarily itself a secondary effect of penicillin, since it involves first an inhibition of mucopeptide formation and secondly some method either for removing part of the wall or so altering its physical properties that it ceases to be rigid; it is doubted whether a simple bursting of the wall at its seams, as a result of expansion of internal contents against a static wall, is a likely mechanism of spheroplast formation. The most convincing evidence, however, for some different effect of high concentrations of penicillin on Gram-negative forms is the work of Meadow (1960). This author found no effect of penicillin on the incorporation of $\alpha\epsilon$-diamino-[^{14}C]pimelic acid into *E. coli* under conditions where growth was strongly inhibited and spheroplasts were formed. This amino acid is usually confined in the cell to the wall mucopeptides. Again the concentration of penicillin necessary was very high (150 μg./ml., unpublished results from Dr Pauline Meadow), and one might conclude from this work that benzylpenicillin at high concentrations has harmful effects on Gram-negative cells other than by inhibiting mucopeptide biosynthesis. However, Nathenson & Strominger (1961) have claimed that benzylpenicillin inhibits the incorporation of tritiated $\alpha\epsilon$-diaminopimelic acid into *E. coli* cell walls by 72%.

The evidence for Gram-negative cells may be confused, but for staphylococci it is completely consistent with the hypothesis that the site of the lethal action of the penicillins is the system responsible for the formation of the cell-wall mucopeptides.

SUMMARY

1. The concentration of benzylpenicillin required to inhibit cell-wall-mucopeptide formation by *Staphylococcus aureus* strain Oxford is of the same order as that required to prevent growth.

2. Benzylpenicillin-resistant cells selected on artificial media from the original culture require

very much higher concentrations of the antibiotic to inhibit cell-wall-mucopeptide formation.

3. The composition of the cell walls of the sensitive and resistant cells is the same.

4. The inhibitory effects upon mucopeptide synthesis by sensitive cells of sodium 6-(2:6-dimethoxybenzamido)penicillin (Methicillin, Celbenin, AB 1241), 6-(α-phenoxypropionamido)penicillanic acid (Broxil) and 6-aminopenicillanic acid are as predicted from their relative potencies tested as antibiotics.

5. Cells made resistant to benzylpenicillin do not require increased concentrations of sodium 6-(2:6-dimethoxybenzamido)penicillin to inhibit mucopeptide synthesis.

6. Sodium 6-(2:6-dimethoxybenzamido)penicillin, like benzylpenicillin, does not inhibit the formation of either proteins or nucleic acids by *Staphylococcus aureus*.

7. Sodium 6-(2:6-dimethoxybenzamido)penicillin inhibits almost completely mucopeptide synthesis by the penicillinase-producing strain *Staphylococcus aureus* 524/SC. The concentration required is similar to that needed by the sensitive strain Oxford.

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The Effects of Corn Oil on the Amounts of Cholesterol and the Excretion of Sterol in the Rat

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Using chicks as experimental animals, Peterson (1951) discovered that the addition of plant sterols to a cholesterol-containing diet decreased the concentration of cholesterol in the plasma. This effect was attributed by Peterson, Shneour, Peek & Gaffey (1953) to an inhibition of the formation of cholesterol esters in the mucosa by plant sterols, including β-sitosterol, present in soya-bean oil. Another mechanism proposed by Glover & Green (1955) for the inhibition of dietary-cholesterol absorption involved a blockage of the sites of absorption of cholesterol in the intestinal mucosa by β-sitosterol and other plant sterols. However, the results of Beveridge, Connell, Mayer & Haust (1958), showing that the concentration of cholesterol in human plasma was lowered by adding corn oil and β-sitosterol to a cholesterol-free diet, cannot be explained fully on the basis of either of the mechanisms cited above. If the limiting factor was ester formation, the absorption of dietary and biliary cholesterol should result in an increase of free cholesterol relative to ester cholesterol in the

intestine when plant sterols are present in the diet. Such an increase has not been reported. If, on the other hand, blockage of the sites of absorption of cholesterol was the limiting factor, the results of Beveridge *et al.* (1958) could be explained only if it was assumed that normally the absorption of biliary cholesterol was considerable. However, only minor amounts of biliary cholesterol appear to be absorbed (cf. Mead & Howton, 1960; Gould & Cook, 1958). Gould (1955) considered that, although the absorption in the intestine of β-sitosterol was limited, nevertheless this sterol might reduce hepatic cholesterol synthesis. However, no confirmation for this hypothesis was given.

In the present work the effect of corn oil on the metabolism of cholesterol was studied by determining the amounts of this constituent in the tissues and organs of rats of different ages. As Haust & Beveridge (1958) found in humans that corn-oil supplementation to a fat-free diet increased the excretion of bile acids, an overall study of the changes in cholesterol metabolism was not considered possible without determining also the amount of faecal sterols.

EXPERIMENTAL AND RESULTS

Two experiments were carried out with male Wistar rats. In the first experiment the object was to observe the effect of corn oil on the cholesterol concentration of the whole animal and of the serum of some of the rats. In the second experiment the concentrations of cholesterol in a number of organs and tissues and in the serum were determined. The excretion of sterols was studied in both experiments.

In Expt. 1 two series of animals were used: series 1, weanling rats (28 days; av. wt. 75 g.); series 2, 5-month-old rats (av. wt. 334 g.). In Expt. 2, 1-year-old rats (series 3; av. wt. 402 g.) were used. The basal low-fat diet (fat content less than 1·4 %) was based on that described by Longenecker (1939) and consisted of (%): casein 20, corn starch 60, sucrose 9, salt mixture 4, agar-agar 2, and dried yeast 5. In addition 2·6 mg. of α-tocopherol acetate, 85 i.u. of calciferol and 600 i.u. of vitamin A acetate, dissolved in paraffin oil, were administered orally once per week to each rat.

In both experiments all animals were first given the low-fat diet for 3 weeks. After this, the experimental groups received supplements of corn oil mixed into the low-fat diet, whereas the control groups continued to receive the low-fat diet alone. In both series 1 and 2 there were four experimental groups, each of five rats, receiving respectively 2 or 10 % supplements of crude or refined corn oil for a period of 5 weeks. In series 3 there was one experimental group of 12 animals which received the basal diet supplemented with 10 % of crude corn oil for 3½ weeks, whereas the control group of 10 rats continued on the basal diet. Although food and water were available *ad libitum* throughout, the food intake as measured in series 3 showed the diets to be nearly isocaloric, as the corn-oil-fed rats ate less food than the control animals (12·2 g. as compared with 13·3 g./rat/day). This difference in intake amounted to 2–3 kcal. out of a total of approximately 45 kcal./day.

At the end of the feeding period food was withheld for 12 hr. The animals of series 1 were then decapitated and those of series 2 and 3 were anaesthetized with ether and then exsanguinated by heart puncture.

The body-cholesterol concentrations of all groups are collected together in Table 1, and Table 2 shows concentrations of cholesterol in a number of organs and tissues of the 1-year-old rats of series 3.

Table 1. *Effect of corn-oil supplements on the body cholesterol of rats at different ages*

Age	Corn-oil supplement	Body cholesterol (mg./100 g. of rat)			Change in cholesterol (mg./100 g. of rat)	
		Free	Total	Ester (total – free)	Free	Ester
Weanling (28 days) (series 1)	None	110	138	28	—	—
	2% crude	135	160	25	+25	−3
	2% refined	134	162	28	+24	0
	10% crude	143	168	25	+33	−3
	10% refined	146	172	26	+36	−2
5 months (series 2)	None	96	127	31	—	—
	2% crude	109	138	29	+13	−2
	2% refined	108	139	31	+12	0
	10% crude	114	146	32	+18	+1
	10% refined	114	144	30	+18	−1
1 year* (series 3)	None	111	137	26	—	—
	10% crude	128	156	28	+17	+2

* Calculated from the individual organs and tissues.

Table 2. *Cholesterol in the tissues of* 1*-year-old rats* (*series* 3) *with and without a* 10% *crude corn-oil supplement*

Organ or tissue	Corn-oil supplement	Tissue cholesterol (mg./100 g. of tissue)			Change in cholesterol (mg./100 g. of tissue)	
		Free	Total	Ester (total – free)	Free	Ester
Aortas	–	120	164	44	—	—
	+	159	198	39	+39	−5
Hearts	–	112	150	38	—	—
	+	127	163	36	+15	−3
Livers	–	202	209	7	—	—
	+	200	227	27	−2	+20
Muscle (rectus femoris)	–	56	65	9	—	—
	+	66	74	8	+10	−2
Adrenals	–	166	1312	1146	—	—
	+	164	1308	1144	−2	−2
Testes	–	92	173	81	—	—
	+	109	176	67	+17	−14
Adipose (perinephric)	–	135	169	34	—	—
	+	144	178	34	+9	0
Intestines	–	116	201	85	—	—
	+	147	252	105	+31	+20
Pelts	–	119	195	76	—	—
	+	114	227	113	−5	+37
Remaining tissues	–	102	113	11	—	—
	+	118	134	16	+16	+5

Table 3. *Serum lipid and cholesterol of rats varying in age and on different corn-oil supplements*

Age	Corn-oil supplement	Serum lipid (mg./100 ml. of serum)	Serum cholesterol (mg./100 ml. of serum)			Change in cholesterol (mg./100 ml. of serum)	
			Free	Total	Ester (total – free)	Free	Ester
5 months (series 2)	None	744	32	116	84	—	—
	2% crude	485	30	82	52	−2	−32
	2% refined	401	29	82	53	−3	−31
	10% crude	249	32	76	44	0	−40
	10% refined	197	25	60	35	−7	−49
1 year (series 3)	None	832	27	113	86	—	—
	10% crude	674	27	98	71	0	−15

Table 4. *Daily excretion of faecal cholesterol and coprosterol by rats varying in age and on different corn-oil supplements*

Age	Corn-oil supplement	Free cholesterol (mg./rat)	Free coprosterol (mg./rat)	Cholesterol plus coprosterol (mg./rat)	Change in cholesterol plus coprosterol	Ratio of cholesterol to coprosterol
Weanling (28 days) (series 1)	None	1·3	15·3	16·6	—	0·085
	2% crude	2·1	15·9	18·0	+ 1·4	0·132
	2% refined	1·7	14·9	16·6	0	0·114
	10% crude	3·5	25·6	29·1	+10·3	0·137
	10% refined	2·9	22·0	24·9	+ 4·9	0·132
5 months (series 2)	None	1·2	12·3	13·5	—	0·098
	2% crude	1·3	12·9	14·2	+ 0·7	0·101
	2% refined	1·2	13·2	14·4	+ 0·9	0·091
	10% crude	3·9	30·4	34·3	+20·8	0·128
	10% refined	3·6	27·6	31·2	+17·7	0·130
1 year (series 3)	None	1·2	11·6	12·8	—	0·103
	10% crude	3·0	27·6	30·6	+17·8	0·109

The serum-cholesterol concentrations of the rats of series 2 and 3 are shown in Table 3.

To determine the weight of faecal coprosterol and cholesterol excreted per day during corn-oil supplementation, the faeces of all animals within each group were collected daily into ethanol and stored in a refrigerator (see Table 4).

The fatty acid composition of the total lipids isolated from serum, tissues and faeces from animals of series 3, as well as that of the crude corn oil, are shown in Table 5. The annotations for the different fatty acids are those used by Farquhar, Insull, Rosen, Stoffel & Ahrens (1959).

Methods

As soon as possible after death, the complete animals (including heads) of series 1 and 2 were rapidly minced and heated in 2 vol. of 95% (v/v) ethanol for 2 hr. at 70° and sealed in jars for storage. The individual tissues and organs of series 3 were similarly treated in a Waring Blendor. The serum was freeze-dried and stored in sealed ampoules. The faeces were also preserved by heating in ethanol.

Within each group the materials thus preserved were combined and the ethanol and water removed on a water bath *in vacuo*. The residues were extracted as described by Gerson, Shorland, Adams & Bell (1959). The same procedure was used for the freeze-dried serum.

Cholesterol in the extracted lipids was determined by the method of Zak, Luz & Fisher (1957) with the ferric chloride–acetic acid–conc. sulphuric acid reagent. To render the method suitable for the determination of cholesterol in the presence of a very large excess of other lipids the solvent systems of the original method were modified and 1 ml. of 1% digitonin solution in ethanol–water (3:1, v/v) was used. The free cholesterol digitonide was washed with 2 ml. of acetone–ethanol (3:1, v/v). To determine total cholesterol a sample containing approximately 1 mg. of lipids was saponified by a modification of the procedure of Sperry & Webb (1950) before the method of Zak *et al.* (1957) was used as for free cholesterol. The samples were dissolved in 1 ml. of ethanol in 15 ml. centrifuge tubes and 1 ml. of ethanolic 0·5N-KOH was added. The tubes were immersed in a water bath at 45–50° for 30 min., and then neutralized (phenolphthalein) with aqueous 30% (v/v) acetic acid and evaporated to a small volume (about 0·1 ml.). The digitonide was precipitated as described above and washed with 2 ml. of ethanol–water (1:1, v/v). Faecal cholesterol and coprosterol were determined as described by Gerson (1960).

All determinations were carried out in quadruplicate and the mean values are quoted. The average standard deviation for quadruplicate sets of determinations was approximately $\pm 0{\cdot}0004$ mg. It was considered that any change involving less than 5 mg. of cholesterol/100 g. of tissue was not significant, owing to the limitation of the methods used and to the experimental design.

The fatty acid composition of the total lipids from tissues, serum and faeces was determined by gas–liquid chromatography in a column (0·5 cm. × 250 cm.) with diethylene glycol adipate polyester (20%, w/w) supported on Celite 545 as the liquid phase. The detector was based on that described by Lovelock, James & Piper (1959) with argon as carrier gas. The methyl esters were identified by comparison of R_F values with those of pure samples and with those given by Farquhar *et al.* (1959).

DISCUSSION

Table 1 shows that the amounts of free and esterified cholesterol of the whole animals of the control groups are not significantly affected by age. However, the increase in the tissue free-cholesterol concentration after the addition of corn oil in the mature animals is approximately half that of the weanlings. Table 1 also shows that the increase in the concentration of body free-cholesterol after a fivefold increase in the amount of corn oil administered is much less than might be expected if this increase was directly proportional to the amount of corn oil added. This result may be partly explained by the large increase in the rate of excretion of cholesterol and coprosterol with the higher amount of corn oil fed and is consistent with the existence of a homeostatic mechanism (cf. Tables 1 and 4).

The determination of coprosterol as well as cholesterol in the faeces was considered necessary since Snog-Kjaer, Prange & Dam (1956) and Coleman & Baumann (1957*a*, *b*) produced strong evidence that coprosterol is a bacterial hydrogenation product of cholesterol. The approximately constant ratio $[0{\cdot}113 \pm 0{\cdot}018(\text{S.D.}):1{\cdot}000]$ of cholesterol to coprosterol in the faecal sterols (see Table 4) indicates the attainment of equilibrium in the hydrogenation of cholesterol. No esterified sterols were found in the faeces.

The amounts of body cholesterol and of daily sterol excretion (Tables 1 and 4) show a considerable increase in cholesterol biosynthesis when corn oil is added to a low-fat diet. This increase is not balanced by the decrease in the amount of serum cholesterol ester (Table 3) in series 2 and 3.

Beveridge, Connell & Mayer (1957), who worked with human subjects on a diet containing fat, obtained similar results. Using a basal fat-free diet containing cholesterol, Klein (1958, 1959) found that the addition of corn oil raised the concentration of serum cholesterol in weanling rats, but in 6-month-old animals the concentration was lowered. Our work (Table 3) also indicates that age affects the changes in the concentration of serum cholesterol when corn oil is added to a low-fat diet. In our experiments the serum of weanling rats was not collected. In the 5-month-old animals the concentration of serum cholesterol was markedly lowered by the addition of corn oil to the diet, but in the 1-year-old rats this decrease was much smaller. Moreover, the age effect is also reflected in the decrease in total plasma lipids. Whereas the addition of 10% of crude corn oil to the basal diet

Table 5. *Fatty acid composition of serum tissue and faecal lipids of 1-year-old rats on a low-fat diet and on the same diet with the addition of 10 % of crude corn oil*

Tr., Trace.

Tissue or organ	Corn-oil supplement	Fatty acids (moles %) 10:0, 12:0	13:0	14:0	15:0 br.	15:0	16:0 iso	16:0	16:1	17:0 br.	17:0	18:0 iso	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	20:4
Serum	–	0·4	—	3·9	—	2·7	—	34·9	7·1	—	1·2	Tr.	13·7	25·5	5·5	Tr.	—	—	—	—	5·1
	+	0·2	—	1·8	—	0·8	—	36·5	1·5	—	1·2	Tr.	15·8	17·6	15·9	Tr.	—	—	—	—	8·7
Aorta	–	—	—	1·2	—	0·2	—	36·8	7·1	—	0·5	0·5	4·0	39·1	6·8	Tr.	Tr.	1·8	—	—	1·5
	+	—	—	1·1	—	Tr.	—	24·4	3·0	—	Tr.	Tr.	4·1	40·0	20·4	—	3·0	3·0	—	—	1·0
Heart	–	0·4	—	0·9	—	0·1	0·1	24·6	4·9	—	0·4	0·4	15·1	28·6	14·6	—	0·6	—	1·7	0·7	7·7
	+	0·4	—	1·5	—	0·2	0·3	24·9	2·9	—	0·8	0·6	9·4	33·7	20·7	—	1·0	—	0·3	0·1	3·2
Liver	–	Tr.	—	1·1	—	—	—	33·7	8·6	—	—	—	16·5	25·5	6·4	—	—	—	—	—	8·2
	+	0·7	—	1·4	—	Tr.	—	33·0	3·5	—	Tr.	Tr.	24·5	25·2	10·9	—	—	—	Tr.	—	1·8
Muscle (rectus femoris)	–	Tr.	—	2·2	—	0·7	—	31·0	10·7	—	0·7	0·7	5·7	35·1	12·1	—	—	1·1	—	—	—
	+	—	—	1·3	—	Tr.	—	22·1	5·4	—	Tr.	Tr.	3·3	43·8	21·5	—	—	2·6	—	—	—
Adrenals	–	—	—	1·6	—	0·5	—	16·7	2·5	—	0·8	—	15·1	57·6	4·1	—	0·9	—	—	—	0·2
	+	—	—	1·0	—	0·5	—	25·0	3·1	—	0·8	—	4·8	51·1	12·6	—	0·5	—	—	—	0·8
Testes	–	—	—	0·8	—	—	—	31·0	9·8	—	Tr.	Tr.	3·1	42·3	10·6	—	—	—	—	—	2·4
	+	—	—	1·3	—	Tr.	—	20·3	12·1	—	Tr.	0·7	2·1	50·0	12·4	—	—	—	—	—	1·3
Adipose (perinephric)	–	—	—	0·8	—	0·3	—	31·2	8·0	—	0·3	0·3	1·1	46·0	10·9	Tr.	0·3	0·7	—	—	—
	+	—	—	0·8	—	—	—	24·7	5·8	—	—	—	1·6	51·9	14·8	0·1	Tr.	0·3	—	—	—
Faeces	–	0·9	1·0	1·6	10·3	7·3	1·9	27·2	2·4	2·4	1·7	0·9	10·0	9·3	3·3	—	1·4	18·4*			
	+	Tr.	0·4	1·3	8·0	9·5	1·5	36·7	2·9	2·5	1·8	0·7	10·6	11·0	4·0	—	0·7	8·4*			
Crude corn oil		—	—	—	—	—	—	10·8	0·1	—	—	—	1·2	37·7	50·0	—	0·2	—	—	—	—

* Acids of unknown constitution with R_F values greater than those for 20:0 acids.

decreased the lipids of 5-month-old rats from 744 to 249 mg./100 ml. of serum, in the 1-year-old animals the lipids were decreased from 832 to only 674 mg./100 ml. of serum. It is remarkable that cholesterol accounts for only approximately 10% of these decreases. The mechanism involved in the lowering of serum-lipid and serum-cholesterol concentrations is at present unknown, as are the reasons for the effect of age.

Table 2 shows the changes in cholesterol of various organs and tissues when a 10% corn-oil supplement is given to 1-year-old rats. As expected in the rats of series 3 the increase in the cholesterol ester of the livers is accompanied by a decrease in the serum cholesterol ester. These results are in harmony with the findings of other workers (cf. Alfin-Slater, Aftergood, Wells & Deuel, 1954; Klein, 1958).

Of particular significance in connexion with studies on atherosclerosis is the observation that in 1-year-old rats whereas 10% corn-oil supplementation produces a 17% decrease of the serum ester cholesterol (Table 3), the free cholesterol of the aortas and hearts increases by 33 and 13% respectively (Table 2). If cholesterol metabolism is linked with the incidence of atherosclerosis then the work now reported indicates that serum cholesterol bears no direct relationship to the amounts of this constituent present in the arteries or in other tissues. Similar results were reported by Merrill (1958), using rat livers. Howard & Gresham (1960) also concluded from their work that 'plasma cholesterol is not necessarily an index of the severity of atherosclerosis and that experiments involving only the determination of plasma cholesterol are of limited value'. The use of serum-cholesterol concentrations may not only give a false picture of the cholesterol concentrations of the tissues, but further obscures the relationships, if any, between cholesterol and the incidence of atherosclerosis. The elevation of sterol excretion, however, affords some indication of major changes in cholesterol biosynthesis in the tissues.

When corn oil is added to the diet the amounts of cholesterol in the tissues and organs are generally raised. In the light of the work of Swell, Trout, Field & Treadwell (1958), the increases in the free and ester cholesterol in the intestinal tissues indicate increased biosynthesis of these constituents. An increase due to the reabsorption of biliary and intestinal cholesterol from the intestinal lumen cannot be entirely ruled out, but since the proportion of free cholesterol in the intestine remains constant at 58% of the total cholesterol (Table 2), the relative increase of free cholesterol, which would be expected if β-sitosterol inhibited esterification as suggested by Peterson *et al.* (1953), does not occur. Further, the blocking of intestinal absorption of cholesterol by β-sitosterol, as suggested by Glover & Green (1955), cannot account for the changes in serum cholesterol described in this paper, since no cholesterol was included in the diet. The increased amounts of cholesterol in the tissues of the rats, together with the increased excretion of sterols in the faeces with corn-oil supplementation found in the present investigation, are consistent with the promotion of biosynthesis of cholesterol but do not exclude the possibility of lowered cholesterol catabolism. The probability that the feeding of corn oil promotes the biosynthesis of cholesterol rather than the decrease of cholesterol catabolism is supported by the recent work of Hill, Webster, Linazasoro & Chaikoff (1960), who have shown that there is increased formation of cholesterol in the liver of rats after the addition of corn oil to a fat-free diet.

Fig. 1 shows a linear increase in free cholesterol with increasing amounts of lipid in the body of the rat from the onset of maturity. On the other hand, the amount of esterified cholesterol is not related to that of the body lipids. The graphs also clearly show the difference in the amounts of free cholesterol between the controls and rats receiving 10% of corn oil.

Table 5 shows that the organ and tissue lipids in the corn oil fed, as compared with the control group, contain more linoleic acid (18:2) in response

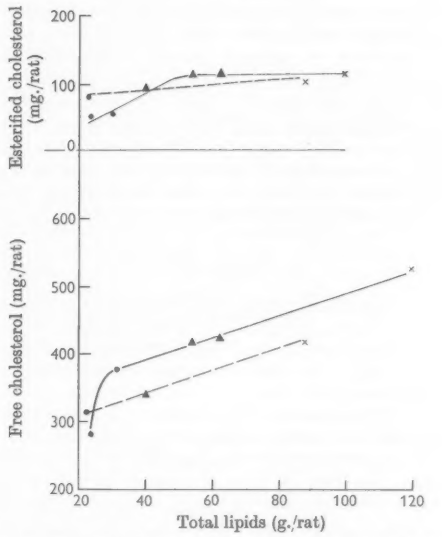

Fig. 1. Relationship of total body lipids to total body cholesterol in rats. —, 10% corn oil; - - -, low fat; ●, series 1; ▲, series 2; ×, series 3.

to the relatively high linoleic acid content (50%) of the dietary fat. As Mead & Howton (1957) demonstrated that linoleic acid (18:2) is converted in the rat into arachidonic acid (20:4), it could be expected that the addition of corn oil to a low-fat diet would result in an increase in the arachidonic acid content of the lipids in the tissues and organs of the rat, such as has been observed by Rieckehoff, Holman & Burr (1949). However, with the exception of the adrenal glands, the addition of corn oil to the diet in the present work resulted in a decrease in arachidonic acid in the lipids of those organs where it was found, i.e. in the aortas, hearts, livers and testes. Apart from the livers this is accompanied by an increase in free cholesterol. In the adrenal glands neither cholesterol nor arachidonic acid changed significantly. On the other hand, in the serum lipids the amount of arachidonic acid was increased and the serum cholesterol diminished by the addition of the dietary corn oil. Our results are consistent with an inverse relationship between the amounts of cholesterol in the tissues and arachidonic acid in the tissue lipids. Klein (1959) has also shown that the elevation of liver cholesterol in rats on feeding cholesterol is accompanied by a decrease in arachidonic acid in the lipids of this organ.

The fatty acid composition of the faecal fat is very unlike that of the tissue and serum lipids, differing largely in the presence of substantial amounts of C_{15} and C_{17} branched and normal fatty acids, as well as higher (C_{20} and above) acids of unknown constitution. The unusual fatty acid composition is perhaps related to the bacterial origin of these lipids. Corn-oil supplementation does not substantially affect the composition of faecal fatty acids, apart from the elevation of the palmitic acid content at the expense of fatty acids, whose methyl esters under the experimental conditions used had retention times greater than that of methyl arachidate.

SUMMARY

1. Supplementation of a low-fat diet with 2 or 10% of corn oil led to an increase in the cholesterol concentrations of the tissues and organs of rats, particularly the hearts, aortas, livers, intestines and muscle.

2. The increases in the cholesterol of the tissues and organs were accompanied by a large decrease in the concentration of serum lipids and a considerable decrease in the concentration of serum cholesterol, these effects being relatively greater in young animals. The decrease in serum cholesterol which accompanied an increase in the overall cholesterol concentrations of the tissues indicates that the serum cholesterol is not a suitable measure of the changes in cholesterol concentration in the animal as a whole. However, some indication of changes in the body cholesterol of rats can be gained by measuring the rate of sterol excretion.

3. There appears to be a definite relationship between body cholesterol and total body lipids in mature rats, the ratio being lower in rats fed on a low-fat diet than those receiving a corn-oil supplement.

4. The fatty acid composition of the tissue and serum lipids reveals an inverse relationship between the concentrations of cholesterol in the tissues and that of arachidonic acid in the lipids, the reason for which is not understood.

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Deacetylcephalosporin C

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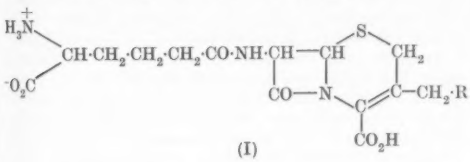
Structure I (R = $CH_3 \cdot CO \cdot O$) has been proposed for cephalosporin C (Abraham & Newton, 1961; Hodgkin & Maslen, 1961). This paper describes the formation and some of the properties of deacetylcephalosporin C (I; R = $\cdot OH$). Deacetylcephalosporin C appeared to be one of the products formed when cephalosporin C was treated with dilute alkali at room temperature. However, under these conditions hydrolysis of the acetoxyl group was accompanied, to some extent, by opening of the β-lactam ring and by other changes in the molecule. The conversion of cephalosporin C into deacetylcephalosporin C in neutral aqueous solution was accomplished, in good yield, by use of an acetyl esterase. Enzymes which hydrolyse esters of acetic acid, but which differ from acetylcholinesterases in that they are not inhibited by low concentrations of eserine, have been found to occur in plants, fungi and animal tissues (Hofstee, 1960). The acetyl esterase used here was that from citrus fruits described by Jansen, Jang & Macdonnell (1947). They reported that acetyl esters were hydrolysed by this enzyme much more readily than esters of other acids, but that *N*-acetyl groups were not hydrolysed.



(I)

METHODS AND EXPERIMENTAL

Elementary analyses were by Weiler and Strauss. Infrared spectra were measured with a Perkin–Elmer double-beam photometer model 21.

Electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14 v/cm. for 2·5 hr.) was carried out as described by Newton & Abraham (1954) in collidine–acetate buffer (0·05 M to acetate), pH 7·0, in pyridine–acetate buffer (0·05 M to acetate), pH 4·5, and in 10% (v/v) acetic acid, pH 2·2. The apparatus used was similar to that of Flynn & de Mayo (1951). Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol–acetic acid–water (4:1:4, by vol.) (Woiwod, 1949), and in propan-1-ol–water (7:3, v/v). Samples of 50–200 μg. were applied to the paper. After chromatography, or after electrophoresis in 10% (v/v) acetic acid, the paper was placed between the viewer and a source of ultraviolet light (230–400 mμ; Corning 9863 filter). Compounds with the cephalosporin C chromophore appeared as dark, light-absorbing, spots.

Bioautographs. After paper electrophoresis or chromatography the paper was dried in air and applied to the surface of a plate of nutrient agar seeded with *Staphylococcus aureus* (Oxford strain, NCTC 6571) or with *Salmonella typhi*, strain 'Mrs S' (Felix & Pitt, 1935). After 20 min. the paper was peeled off and the plate incubated at 37° overnight. Clear zones with no bacterial growth marked the positions of substances active against the test organisms.

Antibacterial activities. These were measured by the hole-plate method, cephalosporin C being used as a standard (Abraham, Newton & Hale, 1954). *Staph. aureus* (NCTC 6571) and *Salm. typhi*, strain 'Mrs S' (Felix & Pitt, 1935) were the test organisms. One unit of activity is contained in 0·1 mg. of cephalosporin C sodium salt dihydrate.

Electrometric titrations. These were carried out at 20° in the manner described by Newton & Abraham (1953).

Citrus acetyl esterase. Partially purified preparations of acetyl esterase were prepared from orange peel by the method of Jansen *et al.* (1947), except that $Na_2S_2O_4$ (0·5 mg./ml.) was added to the press juice, before precipitation with $(NH_4)_2SO_4$, to minimize oxidation. The precipitate obtained with $(NH_4)_2SO_4$ (Jansen *et al.* 1947) was mixed with 0·1 M-sodium oxalate to form a slurry and the latter dialysed against a large volume of 0·1 M-sodium oxalate (adjusted to pH 5·5) at 2° for 60 hr. Most of the solid went into solution and the remaining insoluble material was removed by centrifuging.

About 200 ml. of stock solution of enzyme was thus prepared from the press juice obtained from the peel (1·5 kg.) of 30 Israeli Jaffa oranges. This is referred to as stock solution *A*. About 100 ml. of enzyme solution was also prepared in a similar manner from the press juice obtained from the peel (1·0 kg.) of 40 South African Outspan oranges. This is referred to as stock solution *B*. The stock solutions were stored at 2° and retained their activity for many weeks.

Manometric determination of enzymic hydrolysis. The rates of hydrolysis of cephalosporin C and of triacetin in the presence of citrus acetyl esterase were determined manometrically at pH 7·0 in a Warburg respirometer under conditions similar to those used by Pollock (1952) for determining the activity of penicillinase. The vessels were gassed with CO_2+N_2 (5:95, v/v) and measurements were made at 30°.

Triacetin as substrate. The main compartment of each Warburg vessel contained 2 ml. of an aqueous solution of triacetin and 0·5 ml. of 0·043 M-$NaHCO_3$. The side bulb contained 0·1 ml. of 0·043 M-$NaHCO_3$ and 0·4 ml. of stock solution *A* of acetyl esterase or of a solution made by diluting the latter. Reaction rates were determined with five different concentrations of substrate, ranging from 8 to 130 mM.

Cephalosporin C as substrate. The main compartment of each vessel contained 1·7 ml. of water, 0·3 ml. of stock esterase solution and 0·5 ml. of 0·043 M-$NaHCO_3$. The side bulb contained 0·1 ml. of 0·043 M-$NaHCO_3$ and 0·4 ml. of a solution of cephalosporin C sodium salt. Reaction rates were determined with five different concentrations of substrate, ranging from 1·7 to 28 mM.

Preparative scale hydrolysis of cephalosporin C by acetyl esterase. The enzymic hydrolysis was followed by titration of the acetic acid liberated. In a typical experiment, a preparation of cephalosporin C sodium salt dihydrate (2·18 g., estimated from its antibacterial activity to be about 80% pure) was dissolved in water (5 ml.) and the solution placed in a Pyrex glass vessel fitted with a water jacket kept at 30°. A solution of citrus acetyl esterase (10 ml. of stock solution *A* whose pH had been adjusted to 6·6 with N-NaOH) was warmed to 30° and added. The mixture was stirred with a magnetic stirrer while 0·97 N-NaOH was added from a burette at such a rate that the pH of the solution (glass electrode) remained in the region of 6.6 After 5 hr., when the rate of addition of NaOH had fallen almost to zero, the experiment was stopped. The acid liberated was then equivalent to 0·8 equiv./475 g. of the cephalosporin C preparation used (mol.wt. of cephalosporin C sodium salt dihydrate, 475). The pH of the solution was adjusted to 5·6 with acetic acid and the solution freeze-dried.

Isolation of deacetylcephalosporin C. The freeze-dried product from the enzymic reaction was dissolved in 5 ml. of pyridine–acetate buffer, pH 5·0 (0·3 M to acetate), and applied to a column (30 cm. × 2·9 cm.) of Amberlite XE-58 (120–200 mesh; acetate form). Elution was effected with the same pyridine–acetate buffer, pH 5·0. Fractions (10 ml.) were collected every 10 min. and the ninhydrin colour densities given by 0·1 ml. samples of the fractions were measured by the method of Moore & Stein (1948). A small band of ninhydrin-positive material emerged in fractions 7–15 and a major band was eluted between fractions 80 and 122. Fractions 85–114 were combined and freeze-dried. The freeze-dried powder was dissolved in a minimum amount of water, precipitated with acetone (about 60 ml.), ground to a fine powder under acetone, and washed twice with a further quantity (about 50 ml.) of dry acetone. After having been freed from acetone the powder was dissolved in water (20 ml.), the pH of the solution adjusted to 8·0 by addition of 0·2 N-NaOH, and the solution freeze-dried to yield amorphous deacetylcephalosporin C sodium salt (1·24 g.). The amorphous product was crystallized from aqueous ethanol (Found: C, 37·0; H, 5·5; N, 9·2; S, 7·0; loss of wt. on drying *in vacuo* at 20°, 13·4%. $C_{14}H_{18}O_7N_3SNa,3{\cdot}5H_2O$ requires C, 36·7; H, 5·5; N, 9·4; S, 7·0%; loss of $3{\cdot}5H_2O$, requires 13·7%). It showed $[\alpha]_D^{20}+107°$ in water (*c*, 1·4).

Characterization of acetic acid liberated on enzymic hydrolysis. A solution of cephalosporin C sodium salt (317 mg.) in water (2 ml.) was added to 5 ml. of acetyl esterase (stock solution *A* adjusted to pH 6·5). The mixture was stirred at 30° and 0·2 N-NaOH added at such a rate that the pH remained close to 6·5. After 1·5 hr. the pH remained virtually constant without further addition of NaOH, although the solution showed strong acetylesterase activity when tested by the method of Byrde & Fielding (1955). [About 10 µl. of the solution was spotted on Whatman no. 1 paper and the paper sprayed with diacetylfluorescein (0·01%) in aqueous 50% (v/v) acetone. After keeping the paper for 15 min. at room temperature the spot appeared as a bright green fluorescent zone when viewed in ultraviolet light.] The solution was cooled and its pH adjusted to 2·9 by addition of 2 N-H_2SO_4. It was then freeze-dried and the distillate collected. Electrometric titration of the distillate with 0·2 N-NaOH indicated the presence of an acid with pK_A approx. 4·7. The titre (at pH 8) corresponded to 0·55 equiv./mole of cephalosporin C. This low value was probably due to the retention of some of the acetic acid on freeze-drying. The neutralized distillate was concentrated in a rotary evaporator to about 3 ml., a solution of *p*-bromophenacyl bromide (101 mg.) in ethanol (2 ml.) was added, and the mixture heated under reflux for 70 min. The product which separated on cooling was recrystallized from ethanol–water. It had m.p. 82–84°. When mixed with a sample of authentic *p*-bromophenacyl acetate (m.p. 84°) the m.p. was not depressed.

Alkaline hydrolysis of cephalosporin C. This was carried out at constant pH values by use of a pH Stat (type SBR 2/SBU 1 Titrigraph in conjunction with type TTT 1 automatic titrator, made by Radiometer, Copenhagen). A solution of cephalosporin C sodium salt (20 mg.) in 4 ml. of water was placed in a water-jacketed Pyrex glass vessel. The latter was covered with a polythene lid through holes in which passed a glass electrode, the KCl bridge of a calomel reference electrode, a glass tube carrying a stream of N_2 (which was used to stir the solution and exclude CO_2), and a fine glass tube from a syringe containing 0·1 N-NaOH. After 0·1 N-NaOH had been added from a fine pipette to adjust the pH of the solution to a predetermined value, further 0·1 N-NaOH was added automatically from the syringe at such a rate that the pH remained constant and acid groups liberated by hydrolysis of cephalosporin C were neutralized. The rate of addition of NaOH was recorded. Experiments were carried out at pH 11 and 18° and at pH 9·5 and 38°. After 1·0 or 1·5 equiv. of NaOH/mole of cephalosporin C had been added, Dowex 50X8 (H form) was added to the stirred solution until the pH fell to 6. The solution was concentrated to about 1 ml. and 5 µl. samples

of the concentrate were analysed by paper chromatography in butan-1-ol–acetic acid–water and in propan-1-ol–water.

N-Benzoylation of deacetylcephalosporin C. This was carried out with benzoyl chloride (1 mol.prop.) in a mixture of acetone and aqueous $NaHCO_3$ at 0°, as described by Abraham & Newton (1956) for the benzoylation of cephalosporin C. After removal of the acetone *in vacuo*, the pH of the mixture was adjusted to 2·5. The *N*-benzoyl derivative was extracted with butan-1-ol cooled to −10° (three times with 1 vol.) and immediately re-extracted into water, as the sodium salt, at pH 8·0. The solution of the sodium salt was freeze-dried.

RESULTS

Action of citrus acetyl esterase on cephalosporin C and other compounds

The manometric results obtained with triacetin and cephalosporin C as substrates for the acetyl esterase were analysed by a plot of v against v/s as recommended by Eadie (1942) and by Hofstee (1959). Since the Michaelis–Menten equation can be written in the form:

$$v = V_{max.} - K_m(v/s)$$

this plot gives a straight line with slope $-K_m$ and intercept $V_{max.}$ with the ordinate axis. The plot of v against v/s with cephalosporin C as substrate and stock enzyme solution B is shown in Fig. 1. Values found for Michaelis constants (K_m) with triacetin and cephalosporin C respectively, and relative values of the maximum velocity in the presence of excess of substrate ($V_{max.}$) are shown in Table 1.

No reaction occurred, as judged by manometric experiments, when the preparations of citrus acetyl esterase were mixed with benzylpenicillin, cephalosporin C_e (deacetylcephalosporin C lactone) (Abraham & Newton, 1961), or cephalosporin C_A (pyridine) (I; R = $-C_5H_5N^{+}\cdot$) (Hale, Newton & Abraham, 1961).

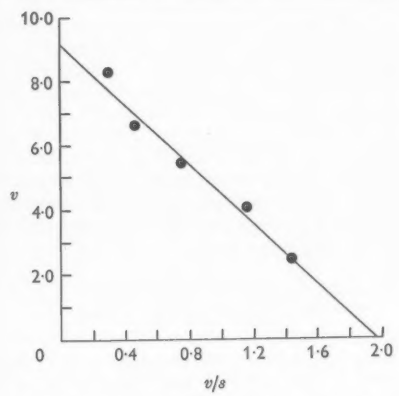


Fig. 1. Plot of v against v/s, where v is the rate of hydrolysis of cephalosporin C sodium salt in the presence of citrus acetyl esterase (μl. of CO_2 evolved/min. from $NaHCO_3$) and s is the concentration of cephalosporin C (mM).

Properties of deacetylcephalosporin C

The elementary composition of the product formed by the action of citrus acetyl esterase on cephalosporin C corresponded with that of a hydrated sodium salt of deacetylcephalosporin C (I; R = OH). Its ultraviolet-absorption spectrum ($\lambda_{max.}$ 261, log ε 3·93), which is shown in Fig. 2, was similar to that of cephalosporin C. Its infrared-absorption spectrum (in Nujol) showed a strong band at 5·77 μ, but, unlike that of cephalosporin C, no band at 5·62 μ or 9·7 μ. Electrometric titration in water indicated the presence of ionizable groups with pK_A values of < 2·5, 3·0 and 9·7 respectively.

Deacetylcephalosporin C sodium salt showed an activity of 2·3 units/mg. against *Staph. aureus* and 1·8 units/mg. against *Salm. typhi*, i.e. about 20% of that of the sodium salt of cephalosporin C. Highly purified preparations of penicillinase from *Bacillus cereus* 569/H and 5/B (Pollock, 1960) had no detectable effect on deacetylcephalosporin C, as measured by the manometric method of Pollock (1952), when used in a concentration 10^4 times that which was sufficient to hydrolyse 2 mg. of benzylpenicillin in about 30 min.

On electrophoresis at pH 7·0 and pH 4·5, deacetylcephalosporin C migrated slightly further towards the anode than cephalosporin C. At pH 2·3 cephalosporin C behaved as though it had no net charge, whereas deacetylcephalosporin C migrated slightly towards the cathode (Table 2). On paper chromatography in propan-1-ol–water or butan-1-ol–acetic acid–water the R_F of deacetylcephalosporin C was significantly less than that of cephalosporin C and the two compounds were well resolved when run together (Table 2). In butan-1-ol–acetic acid–water, however, deacetylcephalosporin C appeared to undergo some degree of change as judged from the appearance of the chromatogram after coloration with ninhydrin.

N-Benzoyldeacetylcephalosporin C showed an activity of about 13 units/mg. against *Staph. aureus* and 0·3 unit/mg. against *Salm. typhi*. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 228 mμ (log ε 4·1) and an inflexion at 265 mμ. Its infrared spectrum (in Nujol) showed a strong band at 5·62. It migrated nearly twice as far as deacetylcephalosporin C towards the anode on electrophoresis at pH 7·0.

In acid solution at room temperature deacetylcephalosporin C was converted into a compound which was indistinguishable by paper electrophoresis and paper chromatography (Table 2) from

Table 1. *Comparison of the behaviour of triacetin and cephalosporin C as substrates for citrus acetyl esterase*

Acetyl esterase from	K_m (mM) for		$V_{max.}$ (μequiv./min./ml. of stock enzyme soln.) for	
	Triacetin	Cephalosporin C	Triacetin	Cephalosporin C
Israeli oranges (stock solution *A*)	32±2	4·7±0·2	2·70±0·2	0·79±0·03
South African oranges (stock solution *B*)	—	4·7±0·2	—	—
Californian oranges	30*	—	—	—

* Value reported by Jansen, Jang & Macdonell (1947).

Table 2. *Behaviour of deacetylcephalosporin C, cephalosporin C, cephalosporin C_c and cephalosporin C_A (pyridine) on paper electrophoresis and chromatography*

The positions of the cephalosporin compounds on paper were detected by coloration with ninhydrin, by viewing the paper in ultraviolet light and by bioautographs (for details see experimental section). Migration towards the anode is denoted by + and towards the cathode by −. In the apparatus used for electrophoresis, buffer was able to evaporate from the paper. At a given potential gradient, more evaporation occurred with collidine–acetate, pH 7·0, than with pyridine–acetate, pH 4·5, because the higher conductivity of the former buffer resulted in a greater production of heat. Differences in the resulting flow of buffer on the paper may have been responsible for the fact that strongly acidic compounds migrated further towards the anode at pH 4·5 than at pH 7·0.

Compound	Migration (cm.) at			$R_{glycine}$ in	
	pH 7·0	pH 4·5	pH 2·3	Butan-1-ol–acetic acid–water	Propan-1-ol–water
Deacetylcephalosporin C	+4·3	+7·2	−1·2	0·57	0·60
Cephalosporin C	+4·2	+6·8	−0·6	0·78	0·77
Cephalosporin C_c	−1·0	−1·7	−4·4	0·85	0·98
Cephalosporin C_A (pyridine)	−1·0	−1·7	—	—	—
Glucose	−1·0	−1·7	−0·6	—	—

deacetylcephalosporin C lactone, cephalosporin C_c. The latter had been previously obtained from cephalosporin C itself by Abraham & Newton (1961). The product from deacetylcephalosporin C was also indistinguishable from cephalosporin C_c with respect to its ultraviolet-absorption spectrum ($\lambda_{max.}$ 257 mμ) and to the change in this spectrum which occurred when the solution was made weakly alkaline (Fig. 2). Its identity with cephalosporin C_c was proved by isolation, the procedure used being the same as that described by Abraham & Newton (1961) for obtaining cephalosporin C_c from cephalosporin C except that lactonization was brought about by treatment with N-HCl for 20 min. The crystalline product from deacetylcephalosporin C showed an infrared-absorption spectrum which was identical with that of authentic cephalosporin C_c, and the two products were reported by Professor Dorothy Hodgkin and Mr R. Diamond to give identical X-ray powder diagrams.

Analysis of reaction mixtures by paper electrophoresis showed that the formation of cephalosporin C_c from deacetylcephalosporin C occurred rapidly at pH values less than 1·0. When deacetylcephalosporin C sodium salt was dissolved in 20 equiv. of N-HCl the reaction was complete in 10 min. Under these conditions very little cephalosporin C_c was formed from cephalosporin C.

Deacetylcephalosporin C was treated with aqueous pyridine under the conditions used by Hale, Newton & Abraham (1961) to demonstrate the formation, on a small scale, of cephalosporin C_A (pyridine) from cephalosporin C. Deacetylcephalosporin C and authentic cephalosporin C_A (pyridine) were easily distinguished by paper electrophoresis at pH 4·5 (Table 2). A bioautograph, made after electrophoresis of the reaction mixture, failed to reveal the presence of any cephalosporin C_A (pyridine), although the formation of the latter in more than 2% yield should have been detected under the conditions used.

Formation of deacetylcephalosporin C by alkaline hydrolysis of cephalosporin C

When an aqueous solution of cephalosporin C sodium salt (13·5 mg./ml.) was kept at 18° and at pH 11·0, 1 equiv. of acid was liberated by hydrolysis in 3·6 hr. Analysis of the reaction mixture by paper chromatography at this stage showed that it contained approximately equal amounts of compounds with R_F values corresponding to cephalosporin C and deacetylcephalosporin C respectively,

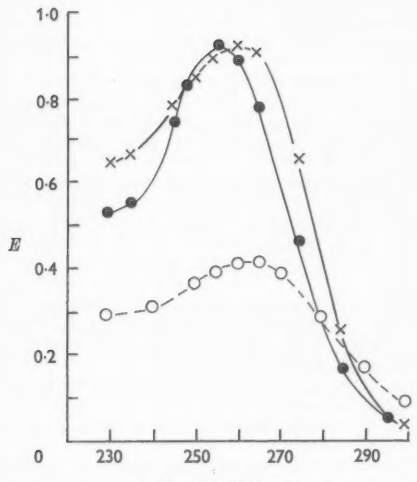


Fig. 2. Ultraviolet-absorption spectra of deacetylcephalosporin C and of products formed from it by treatment with acid and alkali. ×, Deacetylcephalosporin C sodium salt in water; ●, solution obtained by keeping deacetylcephalosporin C sodium salt in 0·25 N-HCl for 30 min. at 20°; ○, solution obtained by keeping deacetylcephalosporin C sodium salt in 0·25 N-HCl for 60 min. and then at pH 10·5 for 35 min. Extinctions are for 0·05 mg. of deacetylcephalosporin C sodium salt (or products formed from 0·05 mg. of the latter)/ml.

together with uncharacterized ninhydrin-positive material. A similar mixture of products was obtained when hydrolysis was carried out at 38° and pH 9·5 for 3·0 hr. From these results it appeared unlikely that deacetylcephalosporin C could be prepared in good yield by simple alkaline hydrolysis of cephalosporin C.

DISCUSSION

The enzymic hydrolysis of a number of different esters ($R{\cdot}CO_2X$) appears to involve the preliminary formation of an enzyme–substrate complex ($R{\cdot}CO_2X$–E), followed by acylation of the enzyme (E) to yield R·CO–E and subsequent hydrolysis of the acylated enzyme (Gutfreund & Hammond, 1959; Hofstee, 1960).

$$E + R{\cdot}CO_2X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [R{\cdot}CO_2X\text{–}E] \xrightarrow{k_2} R{\cdot}CO\text{–}E$$
$$+ XOH \xrightarrow{k_3} E + R{\cdot}CO_2H + XOH$$

If this were so in the hydrolysis of cephalosporin C and triacetin by citrus acetyl esterase the Michaelis constants, K_m (determined, for example, from a plot of v against v/s), should be related to the different velocity constants by the expression of Gutfreund & Sturtevant (1956):

$$K_m = (K_m)_1 \frac{k_3}{k_2 + k_3}$$

where

$$(K_m)_1 = \frac{k_{-1} + k_2}{k_1}$$

and the overall velocity constant, k_0, is given by

$$1/k_0 = 1/k_2 + 1/k_3$$

For the hydrolysis of two acetyl esters k_3 should be the same. The determined Michaelis constant, K_m, with triacetin as substrate is about seven times that with cephalosporin C. According to the above scheme the value assigned to $(K_m)_1$ with triacetin would thus be $7(k_2+k_3)/(k_2'+k_3)$ times as great as with cephalosporin C, where k_2 and k_2' are corresponding velocity constants for triacetin and cephalosporin C respectively. The finding that $V_{max.}$ (i.e. $k_0[E]$) is about 3·4 times as great for triacetin as for cephalosporin C would imply that $k_2 > k_2'$. Hence $(K_m)_1$ with triacetin would be at least seven times that with cephalosporin C but not significantly greater than seven times if acylation were the rate-determining process in each case, that is, if $k_2 \ll k_3 \gg k_2'$.

A number of properties of deacetylcephalosporin C, including its ultraviolet-absorption spectrum and its ready conversion into cephalosporin C_c, indicated that the enzymic removal of the acetyl group from cephalosporin C had been brought about without other changes having occurred in the molecule. The failure of deacetylcephalosporin C to form cephalosporin C_A (pyridine) was to be expected if the formation of the latter from cephalosporin C involved displacement of the acetoxyl group, as suggested by Hale *et al.* (1961). The presence of a band at 5·77 μ and the absence of a band at 5·62 μ in the infrared spectrum of deacetylcephalosporin C sodium salt, however, were unexpected. In the infrared spectrum of cephalosporin C a band at 5·62 μ is attributed to the C=O of the β-lactam ring and a band at 5·77 μ to the C=O of the acetoxyl group. The displacement of the lactam C=O band from its normal position in deacetylcephalosporin C sodium salt may be due to effects which occur in the solid state. This view is consistent with the finding that the infrared spectrum of *N*-benzoyldeacetylcephalosporin C showed a strong band in the expected position at 5·62 μ and no band at 5·77 μ.

SUMMARY

1. Citrus acetyl esterase catalyses the hydrolysis of the *O*-acetyl group in cephalosporin C. The Michaelis constant for the reaction was found to be 4·7 mM.

2. Deacetylcephalosporin C has been isolated as a crystalline sodium salt. It resembles cephalosporin C in a number of its properties, including its ultraviolet-absorption spectrum, but is more readily converted into the lactone, cephalosporin C_c, in acid solution.

3. Deacetylcephalosporin C shows about 20% of the activity of cephalosporin C against *Staphylococcus aureus* (Oxford strain) and *Salmonella typhi*. It is highly resistant to hydrolysis by purified preparations of penicillinase from *Bacillus cereus* strains 569/H and 5/B.

4. Deacetylcephalosporin C and cephalosporin C may be distinguished by paper chromatography.

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Studies on Human Urinary and Pituitary Gonadotrophins

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The interest in human gonadotrophic hormones derives from the necessity for their accurate measurement in body fluids and from the prospect of their therapeutic usefulness. The separation of these hormones from urine and from pituitary glands and the comparison of certain of their biological, physical and immunological properties form the subjects of this paper.

METHODS

Preparation of gonadotrophins

From urine. Urinary gonadotrophins have been prepared by two different methods.

(i) Pooled urine of postmenopausal women was extracted by the benzoic acid–tungstic acid method described by Butt (1958). Material soluble in water was adsorbed on diethylaminoethylcellulose and was then eluted in 0·5M-ammonium acetate. After precipitation from 3 vol. of ethanol the material was redissolved in water and fractionated by chromatography on a column of diethylaminoethylcellulose. Material eluted in 0·2M-ammonium acetate was collected and reprecipitated from 3 vol. of ethanol, washed with ethanol and ether and dried (Butt, Crooke & Cunningham, 1959).

(ii) A generous supply of urinary gonadotrophin from postmenopausal women (HMG-J5) was given to us by Dr S. G. Johnsen (Statens Seruminstitut, Copenhagen). It had been prepared by Johnsen's (1955) method and was further fractionated by chromatography on a column of calcium phosphate by the procedure described by Butt, Ingram & Round (1956), except that the calcium phosphate was prepared by a method described by Main, Wilkins & Cole (1959). Calcium phosphate was precipitated by

adding 100 ml. of 0·5 M-calcium chloride solution dropwise with stirring to 120 ml. of 0·5 M-phosphate buffer, pH 6·7. Stirring was continued for 1 hr. and then the precipitate was washed by decantation with water. Water (600 ml.) was then added and the mixture was transferred to a large conical flask. A few drops of phenolphthalein were added and sufficient ammonia (sp.gr. 0·88) to turn the indicator pink. The suspension was boiled for 30 min., more ammonia being added if necessary to maintain the pink colour. Finally the precipitate was thoroughly washed with water and suspended in 50 ml. of 0·01 M-disodium phosphate.

From human pituitary glands. Three human pituitary-gland preparations were kindly supplied by Dr A. Korner (University of Cambridge, Department of Biochemistry). These were (i) an acetone-dried extract of pituitary glands, (ii) an extract of the powder in potassium chloride at pH 5·5, and (iii) a by-product from the process used for extracting growth hormone. This latter material, HGH.1/3, was prepared as previously described (Butt, Crooke, Cunningham & Evans, 1961).

The powders were subsequently treated in this Laboratory. The first steps were essentially the same as those described by Steelman, Segaloff & Andersen (1959). The acetone-dried powder was extracted in 10% ammonium acetate–ethanol (60:40, v/v) and then precipitated by addition of ethanol until the concentration was 80% (v/v). From this stage the subsequent steps were the same for all three preparations (Fig. 1). Amounts of 100–200 mg. were applied to columns (20 cm. × 1 cm.) of carboxymethylcellulose in 0·01 M-ammonium acetate, pH 6·1. Fraction CM1 was not adsorbed under these conditions and fraction CM2, which was adsorbed, was eluted in 1 M-ammonium acetate, pH 6·1. CM1 was further fractionated on a column of diethylaminoethylcellulose (10 cm. × 1 cm.) by the method described for urine by Butt *et al.* (1959). The fraction eluted in 0·2 M-ammonium acetate was then put directly through a column of calcium phosphate (10 cm. × 1 cm.) and fractions eluted in 1 mM- and 0·02 M-disodium phosphate were collected, dialysed and reprecipitated by adding 5 vol. of ethanol.

CM2 was refractionated on carboxymethylcellulose (20 cm. × 1 cm.) by stepwise elution in ammonium acetate (0·01–1·0 M). Gonadotrophin was detected only in the fraction eluted in 0·1 M-ammonium acetate.

In all chromatographic work the elution of protein was detected by observing the ultraviolet absorption of the eluate at 280 mμ in a Unicam spectrophotometer SP. 500.

Ultracentrifugal studies

These were performed by Dr D. R. Stanworth and Miss Ratcliffe of the University of Birmingham, Department of Experimental Pathology. A Spinco analytical model E was used. The proteins were dissolved in barbitone buffer (pH 8·6, *I* 0·05; 0·2 M-sodium chloride) and centrifuged at 60 000 rev./min. for 160 min.

Biological assay

Two assays with mice have been fully described previously (Butt *et al.* 1961). They are (*a*) the uterine-weight method, which is believed to measure the combined effect of follicle-stimulating hormone and interstitial-cell-stimulating hormone, and (*b*) the augmentation method, which is believed to be relatively specific for follicle-stimulating hormone.

Human chorionic gonadotrophin was measured by the method of Loraine (1950). This depends upon the increase in weight of the prostate gland in intact immature rats.

The results were calculated by the methods of Gaddum (1953) and Borth (1960). The standard used was the international reference preparation, HMG 24. Relative potencies were calculated in terms of this standard with laboratory substandards calibrated against it. The ratio of the potencies of any preparation in terms of standard by two methods of assay has been termed the 'index of discrimination' (Gaddum, 1955). If this ratio is less than 2·0, or the 95% fiducial limits of error overlap, the preparation is not considered to differ qualitatively from the standard (Cunningham & Brown, 1958).

Immunological techniques

Production of antisera. Bentonite (British Drug Houses Ltd.) (approx. 1 g.) was suspended in 100 ml. of 0·9% sodium chloride solution. Coarse particles were allowed to

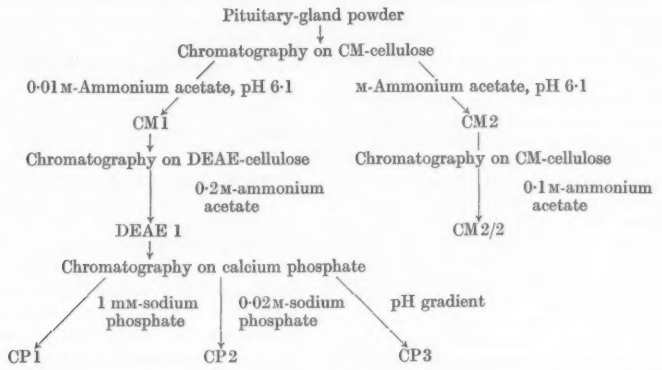

Fig. 1. Scheme for the chromatographic separation of pituitary-gland powders with carboxymethyl (CM)-cellulose and diethylaminoethyl (DEAE)-cellulose and calcium phosphate (CP).

settle and were then separated by decantation and discarded. Gonadotrophin was mixed with an equal weight of the suspended bentonite and was administered intravenously to rabbits (sandylops).

Amounts of 0·5 mg. of gonadotrophin in 2 ml. of suspension were given twice a week for 3 weeks or more. Rabbits were bled at intervals after the last injection and it was found that the highest activity in the antiserum occurred between 6 and 12 days afterwards. Subsequently therefore antiserum was withdrawn 7 days after the final injection as a routine. Usually satisfactory results were obtained after treatment for only 3 weeks.

Examination of the antisera. (1) Biological activity. Antiserum (0·025–0·4 ml.) was injected intraperitoneally into mice in two equal doses on the first and second days. Gonadotrophin (equivalent to 1–3 mg. of the standard) was injected subcutaneously in four equal doses, each in 0·5 ml., on the second and third days. On the fourth day the mice were killed and the uteri dissected and weighed. Control mice were given sodium chloride solution or serum which had been withdrawn from rabbits before immunization, instead of antiserum. This was followed by the same dose of gonadotrophin as was given to the experimental mice. The effect of antiserum on the activity of human chorionic gonadotrophin was examined by the rat prostate-gland method. The antiserum (0·025–0·4 ml.) was injected intraperitoneally on the first and second day and the human chorionic gonadotrophin (1·5 and 4·5 i.u.) subcutaneously on the second, third and fourth days, the total dose being in 1 ml. On the fifth day the prostate gland and surrounding structures were dissected and fixed in Bouin's fluid for 24 hr. Then the prostate gland was dissected and weighed.

(2) Agar-gel method. Solutions of 1% (w/v) agar (Ionagar, Oxo Ltd.) were prepared in several buffers. These were 0·1 M-sodium acetate in 0·9% sodium chloride, adjusted with acetic acid to pH 5·5 and pH 6·5, and phosphate buffer (0·1 M in 0·9% NaCl) at pH 7·6. Antigen and antibody were placed in wells made in the gel, of 5 mm. diameter, with the centres 8 mm. apart. Diffusion into the gel was allowed to proceed overnight, or longer in some experiments, at room temperature. If a permanent record of the experiment was required the gels were washed in a solution of 1% formalin in phosphate buffer (pH 7·6) for 20 min. and then in running water for several hours. Next they were stained in a 0·2% solution of chlorazol black (Gurr and Co. Ltd.) for about 30 min. and washed in water until the background was pale. The gel was then transferred to a glass slide and dried.

(3) Red-cell-haemagglutination technique. Sheep cells preserved in Alsever solution were obtained from Wellcome Research Laboratories Ltd. In early experiments the cells were tanned and coated with antigen exactly as described for human growth hormone (Read & Bryan, 1960), 100–500 μg. of antigen being used for 6 ml. of red-cell suspension. In later experiments the modification of Ling (1960) was used. Red cells were washed three times with sodium chloride solution and were then treated with pyruvic aldehyde (25% solution, L. Light and Co. Ltd.). Reagent (1·6 vol.) was added to sodium chloride solution (3·0 vol.) and the pH was brought to approximately 7·0 with 10% sodium carbonate solution. Phosphate buffer (0·15 M, pH 8·0, 0·7 vol.) and red-cell suspension (1 vol. of 50% suspension) were then added to the mixture and stored at 4° for 2 days with occasional mixing. The cells were thoroughly washed with sodium chloride solution and stored as a 10% suspension containing 0·1% of azide as preservative.

Antigen was then attached to the cells. A portion (2 ml.) of the 10% suspension of cells was centrifuged and the supernatant discarded. The cells were washed once with dilute buffer (sodium chloride–water–phosphate, 0·15 M, pH 6·0; 10:10:1, by vol.) and then suspended in the same buffer (1·5 ml.) containing 100–500 μg. of antigen. After heating at 50° for 1 hr. the cells were centrifuged and washed three times with sodium chloride solution containing rabbit serum (0·5%) and finally were suspended in 10 ml. of sodium chloride solution containing rabbit serum (1%, v/v) and 0·1% of azide.

Antiserum was heated at 56° for 30 min. and was then absorbed with sheep red cells as described by Read & Bryan (1960). A series of dilutions in serum–sodium chloride solution (1%, v/v) was made and 0·2 ml. was pipetted into Perspex agglutination trays (R. B. Turner and Co. Ltd.). A portion (0·2 ml.) of serum–sodium chloride solution was added to each well, followed by two drops (0·05 ml. approx.) of red-cell suspension. After mixing the cells were covered and allowed to settle overnight. If no haemagglutination occurred the cells sank to the bottom of the well, forming a discrete button; if haemagglutination occurred the cells spread out evenly over the bottom of the well.

RESULTS

Urinary gonadotrophin

Human menopausal gonadotrophin prepared by the benzoic acid–tungstic acid method followed by fractionation on diethylaminoethylcellulose was assayed by the mouse uterine-weight and augmentation methods. The doses of this preparation were 47·5 and 95·0 μg. in the uterine-weight assay and 110·0 and 220·0 μg. in the augmentation assay. The results (Table 1) showed that the two assays gave approximately the same result as we have found previously for this method of extraction (Butt *et al.* 1959). Ultracentrifugal analysis showed that the main component (82% of the total protein) had sedimentation coefficient ($S_{20,w}$) 2·6. There was one other recognizable component with a higher $S_{20,w}$ which could not be calculated accurately.

HMG-J 5 was assayed after fractionation on a column of calcium phosphate (10 cm. × 1 cm.). Active material was obtained in the fractions eluted in 1 mM-disodium phosphate (CP 1) and in 0·02 M-disodium phosphate (CP 2). In the uterine-weight assay doses of 10·0, 20·0 and 40·0 μg. of CP 1 and 20·0 and 40·0 μg. of CP 2 were used. In the augmentation assay doses of 2·5, 5·0 and 10·0 μg. of CP 1 and 10·0, 20·0 and 40·0 μg. of CP 2 were used. Both fractions were considerably more potent in the augmentation assay than in the uterine-weight assay and appeared therefore to be qualitatively different from the standard. Ultracentrifugal investigations indicated that CP 1

contained a major component (90·9 %) with $S_{20,w}$ 2·0, and CP 2 contained 64 % of a component with $S_{20,w}$ 1·64. There was one minor component with a higher $S_{20,w}$ recognized in each fraction.

Pituitary-gland gonadotrophin

Assay of initial materials. The potencies of the three starting materials (the acetone-dried powder, the potassium chloride extract and HGH. 1/3) are given in Table 2. The potencies of the potassium chloride extract and HGH. 1/3 were considerably greater than the acetone-dried powder; however, whereas there was no significant qualitative difference in the results by the two methods of assay when applied to the potassium chloride extract, there was a difference in HGH. 1/3. This was considerably more potent in the augmentation assay than in the uterine-weight assay (index of discrimination 2·5) with no overlap of fiducial limits, which suggests that during its preparation some interstitial-cell-stimulating hormone had been removed from the follicle-stimulating hormone.

Fractionation procedures. The fractions CM 1 and CM 2 were first prepared from the potassium chloride extract. Details of the assays are given in Table 3.

The index of discrimination in the assay of CM 1 was greater than 1 and in the assay of CM 2 was considerably less than 1. This suggests that CM 1 was relatively richer in follicle-stimulating hormone than the standard and conversely CM 2 was richer in interstitial-cell-stimulating hormone. The total recovery was reasonably good and the procedure was therefore considered to be a useful initial step. Because of shortage of potassium chloride extract further batches of CM 1 and CM 2 were prepared from HGH. 1/3. These were then used for subsequent fractionations.

Fractionation of CM 1. Three separate batches of CM 1 were fractionated on diethylaminoethylcellulose. DEAE 1 was eluted in 0·2 M-ammonium acetate and contained nearly all the detectable activity. Material left on the column was eluted in 0·5 M-ammonium acetate (at pH 9·5) made up in

Table 1. *Potency of urinary gonadotrophin*

The first figure in the design of each experiment gives the number of doses of unknown, the second the number of doses of standard. In each assay here and in the subsequent Tables there was no significant (P 0·05) divergence from parallelism in the log dose–response lines, and where more than two points were used no significant departure from linearity. The index of precision (λ) was between 0·14 and 0·23 and the number of animals used in these assays was between 13 and 37. The index of discrimination is the result for the augmentation assay divided by the result for uterine-weight assay.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
DEAE 1	2:2	19 (13–28)	2:2	15 (10–27)	0·8
HMG-J5 CP1	3:2	107 (72–207)	3:2	345 (225–520)	3·2
HMG-J5 CP2	2:2	23 (12–33)	2:2	116 (85–158)	5·0

Table 2. *Potency of pituitary-gland gonadotrophin*

λ was between 0·11 and 0·28. The number of animals used in these assays was between 12 and 28.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)
Acetone-dried powder	3:2	15 (11–19)	3:2	14 (9·5–21)
Potassium chloride extract	3:2	59 (37–255)	2:2	63 (30–192)
HGH. 1/3	2:2	74 (63–86)	3:2	183 (125–285)

Table 3. *Fractionation of pituitary-gland gonadotrophin on carboxymethylcellulose*

Each assay was at least 2:2-point design. λ was 0·23–0·28. The number of animals used in the assays was between 16 and 28.

	Potency (mg. of standard/mg.)			Total (mg. of standard)		Recovery (%)	
Preparation	Uterine-wt. assay	Augmentation assay	Index of discrimination	Uterine-wt. assay	Augmentation assay	Uterine-wt. assay	Augmentation assay
Potassium chloride extract	59 (37–255)	63 (30–192)	1·1	2600	2750	—	—
CM 1	113 (46–299)	183 (84–355)	1·6	1140	1820	60	72
CM 2	78 (27–220)	28 (4–57)	0·36	410	146		

0·2 M-sodium chloride. This fraction accounted for less than 0·5 % of the original activity of the CM 1 put on the column. Details of the assay on DEAE 1 are given in Table 4. The potencies of the three batches were in reasonable agreement but in each the index of discrimination was high, suggesting that DEAE 1 was rich in follicle-stimulating hormone compared with the standard.

Ultracentrifugal investigations indicated that the first batch contained one recognizable component ($S_{20,w}$ 3·95) (Fig. 2*a*). After 144 min. sedimentation there was a suggestion of some heterogeneity in the form of a slight shoulder on the trailing edge. The other two batches each contained a component with similar $S_{20,w}$ (71 % in the one and 67 % in the other) and in addition a second component with lower $S_{20,w}$ (2·6–3·0).

Fractionation of DEAE 1 on calcium phosphate. Details of the assays of CP 1 and CP 2 fractions are given in Table 5. In the uterine-weight assay doses of 0·5 and 1·0 μg. of CP 1 and 2·0, 4·0 and 8·0 μg. of CP 2 were used. In the augmentation assay doses

Table 4. *Potency of DEAE 1 fraction*

λ was between 0·10 and 0·20. The number of animals used in each assay was between 13 and 22.

Batch no.	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
1	3:2	86 (65–144)	3:3	240 (180–320)	2·8
2	2:2	81 (72–129)	2:2	220 (144–525)	2·7
3	3:2	83 (47–290)	2:2	195 (132–478)	2·4

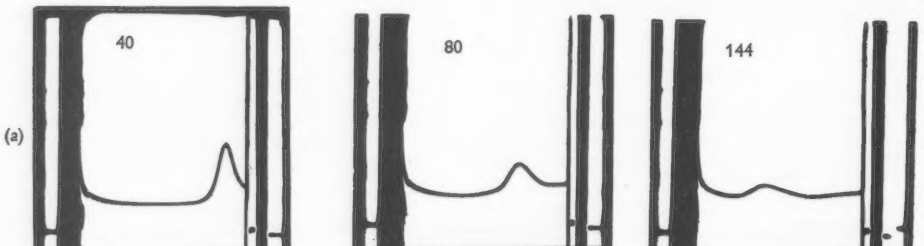

Fig. 2. Ultracentrifugal patterns obtained with gonadotrophins prepared from human pituitary glands. Protein concentration in each was approx. 1 %. Temp. 20°. Barbitone buffer, pH 8·6, I 0·05, in 0·2 M-NaCl. (*a*) Pituitary-gland DEAE 1; (*b*) pituitary-gland CP 2. Numbers refer to time (min.).

Table 5. *Potency of CP 1 and CP 2 fractions*

λ was between 0·11 and 0·28. The number of animals used in each assay was between 29 and 40.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
CP 1	2:2	1318 (999–1722)	2:2	3002 (1938–6956)	2·2
CP 2	3:2	271 (194–391)	3:2	1014 (753–1555)	3·7

Table 6. *Potency of CM2 and CM2/2 fractions*

λ was between 0·12 and 0·21. The number of animals used was between 18 and 47.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
CM2	2:2	49 (41–68)	2:2	30 (12–35)	0·62
CM2/2	2:2*	79 (22–134)	2:2	20 (11–28)	0·25

* In the weighed uterine response the log dose–response line for fraction CM2/2 had a lower slope than the standard line and it was difficult to assemble a satisfactory quantitative assay. It was possible to obtain a valid assay by a probit method (Brown, 1955), a uterine weight of 10 mg. being classified as a positive response, and these results have been calculated by such a method. λ was 0·26 and the number of animals used was 42.

Table 7. *Relative potencies of pituitary-gland fractions*

λ was between 0·15 and 0·27. The number of animals used in each assay was between 29 and 60.

Preparation	Design	Uterine-wt. assay (mg. of CM2/mg.)	Augmentation assay (mg. of CM2/mg.)	Index of discrimination
CP1	3:2	13·4 (10·6–17·8)	81·3 (56·3–159·4)	6·1
CP2	2:2	4·0 (3·0–5·4)	33 (19–130)	8·25

of 0·5 and 1·0 μg. of CP1 and 1·0, 2·0 and 4·0 μg. of CP2 were used. The potency of each was considerably greater than DEAE1 and in CP2 the index of discrimination had increased further. When examined in the ultracentrifuge CP2 appeared to be homogeneous with $S_{20,w}$ 3·07 (Fig. 2*b*), whereas CP1 contained a major component (64%) with $S_{20,w}$ 1·64 and a minor component (36%) with a higher $S_{20,w}$.

Fractionation of CM2. CM2 was fractionated by chromatography on carboxymethylcellulose. The material eluted in 0·1 M-ammonium acetate (CM2/2) contained all the detectable gonadotrophin. The doses of CM2 were 16·0 and 32·0 μg. in the uterine-weight assay and 32·0 and 64·0 μg. in the augmentation assay, and the doses of CM2/2 were 3·0, 6·0 and 12·0 μg. in the uterine-weight assay and 40·0 and 80·0 μg. in the augmentation assay. Details of the assays are given in Table 6.

In contrast with the results with the CM1 fraction the index of discrimination was less than 1·0. The shape of the log dose–response curve in the uterine-weight method for CM2/2 was different from standard, the slope of the line being significantly less than that of the standard (P 0·01). The result was therefore calculated by a probit method (Brown, 1955), by which it was possible to obtain a valid assay. Ultracentrifugal examination of CM2/2 indicated a major component (95%) with $S_{20,w}$ 4·17.

Comparison of biological activities. The figures suggested that the fractions CP1 and CP2 were relatively rich in follicle-stimulating hormone and that CM2 was rich in interstitial-cell-stimulating hormone when compared with the standard. They were therefore compared directly with one another and the results are given in Table 7. The index of discrimination increased to 6·1 when CP1 was assayed against CM2 and to 8·25 when CP2 was assayed against CM2.

Table 8. *Recovery of gonadotrophin during fractionation of HGH.1/3*

The total amount of starting material (HGH.1/3) and the amounts recovered in CP1, CP2 and CM2 were measured by the two methods of assay. The total percentage recovered in the three fractions is given in the last two columns.

	Total (mg. of standard)		Recovery (%)	
	Uterine-wt. assay	Augmentation assay	Uterine-wt. assay	Augmentation assay
HGH.1/3	42 300	105 000	—	—
CP1	17 600	40 500		
CP2	2 950	11 000	67·5	53
CM2	8 000	4 270		

Recovery of biological activity. The recovery of biological activity in fractions CP1, CP2 and CM2 was good in view of the number of steps involved. The overall yield in the three fractions combined was over 50% of the original activity in HGH.1/3. The results are shown in Table 8. The recovery of CM2/2 from CM2 was, however, only 22·5% by the uterine-weight assay and 10·7% by the augmentation assay. These results are given in Table 9.

Immunological investigations

Rabbit antisera were raised against the DEAE1 fraction from both a urinary and a pituitary-gland extract. A total dose of 19 mg. of the

urinary and 5 mg. of the pituitary-gland preparation was used.

Experiments in vivo. Both antisera were effective in inhibiting the activity of urinary and pituitary-gland gonadotrophins. Three different urinary DEAE 1 fractions and two different pituitary-gland DEAE 1 fractions were investigated. They were given in doses equivalent to 1·5 and 3·0 mg. of the standard. In each experiment the potency was reduced by more than 50 % by 0·1 ml. of antiserum. The pituitary-gland antiserum was also examined for effectiveness against pituitary-gland CP 1, CP 2 and CM 2 used at doses equivalent to 1 and 2 mg. of the standard. Again 0·1 ml. of antiserum reduced the potency by more than 50 %.

In contrast with these results both antisera were ineffective against human chorionic gonadotrophin in rats. Doses of 0·025–0·4 ml. of each antiserum caused no reduction in the weight of the prostate gland of rats given 1·5 and 4·5 i.u. of human chorionic gonadotrophin compared with control animals given human chorionic gonadotrophin alone ($P > 0·2$). Higher doses than 0·4 ml. caused toxic effects. These results are summarized in Table 10.

Other investigations of antisera. By the double-diffusion technique in agar gel at least two precipitin lines were observed between the antisera to urinary DEAE 1 and urinary DEAE 1 antigen (Fig. 3). The pituitary-gland DEAE 1 antigen gave only one line against this antiserum. The fractions CP 1 and CP 2, however, gave no reaction and the antigen responsible for the precipitin line was found to be in CP 3, which was eluted from the calcium phosphate column by a pH gradient with 0·02 M-disodium phosphate and N-sodium hydroxide. Fraction CP 3 was biologically inert.

The antiserum to pituitary DEAE 1 gave no precipitin lines under any conditions tried. Buffer of pH 5·5–7·6 and widely differing concentrations of antigen and antibody were used in these experiments.

Reactions were obtained with the red-cell-haemagglutination technique. Cells treated with pyruvic aldehyde were coated with pituitary-gland DEAE 1, 100 μg./2 ml. of the 10 % suspension of cells being used. Haemagglutination occurred in those mixtures in which the final concentration (the titre) of the antiserum to pituitary-gland DEAE 1 fraction was not less than 1 in 1500. Titres as high as 1 in 10 000 were obtained with the antiserum to the urinary DEAE 1 fraction. The agglutination was inhibited by human menopausal gonadotrophin and by pituitary-gland gonadotrophin. For these experiments it was found convenient to use a dilution of antiserum of one-half of the titre (i.e. 1 in 750 for a titre of 1 in 1500). The gonadotrophin was dissolved in sodium chloride solution (usually 1 mg./ml.) and doubling dilutions were made. A portion (0·2 ml.) of each dilution was added to 0·2 ml. of the diluted antiserum, followed by 0·05 ml. of the red-cell suspension. Inhibition of the agglutination occurred in those mixtures in which sufficient gonadotrophin had been added to neutralize the antibody. An example of this reaction is shown in Table 11. In the preliminary titration of the antisera the last agglutination occurred in the mixture containing a dilution of 1 in 6400 of the antiserum to urinary and 1 in 1152 of the antiserum to pituitary-gland gonadotrophin. A dilution of 1 in 576 of antiserum to pituitary-gland gonadotrophin was chosen in the inhibition experiment and it was found that a varying degree of inhibition occurred with different preparations of human menopausal gonadotrophin. The inhibition was not specific for human menopausal gonadotrophin, however, since human chorionic gonadotrophin also inhibited the reaction.

Agglutination and inhibition reactions have also been demonstrated with red cells coated with other pituitary-gland fractions including CP 1 and CM 2.

Table 9. *Recovery of gonadotrophin during fractionation of CM 2*

The total amount of starting material (CM 2) and the amount recovered in CM 2/2 were measured by the two methods of assay. The percentage recovered in CM 2/2 is given in the last two columns.

	Total (mg. of standard)		Recovery (%)	
Preparation	Uterine-wt. assay	Augmentation assay	Uterine-wt. assay	Augmentation assay
CM 2	2600	1380	—	—
CM 2/2	590	145	22·5	10·7

Table 10. *Inhibition of gonadotrophin by antisera*

+, Antiserum was effective in reducing the activity of the gonadotrophin; −, antiserum was ineffective at the highest dose tested. Dose of antiserum used was between 0·025 and 0·4 ml.; more than 0·4 ml. was usually toxic.

Antiserum	Human menopausal gonadotrophin	Pituitary gonadotrophin	Human chorionic gonadotrophin
Urinary DEAE 1	+	+	−
Pituitary-gland DEAE 1	+	+	−

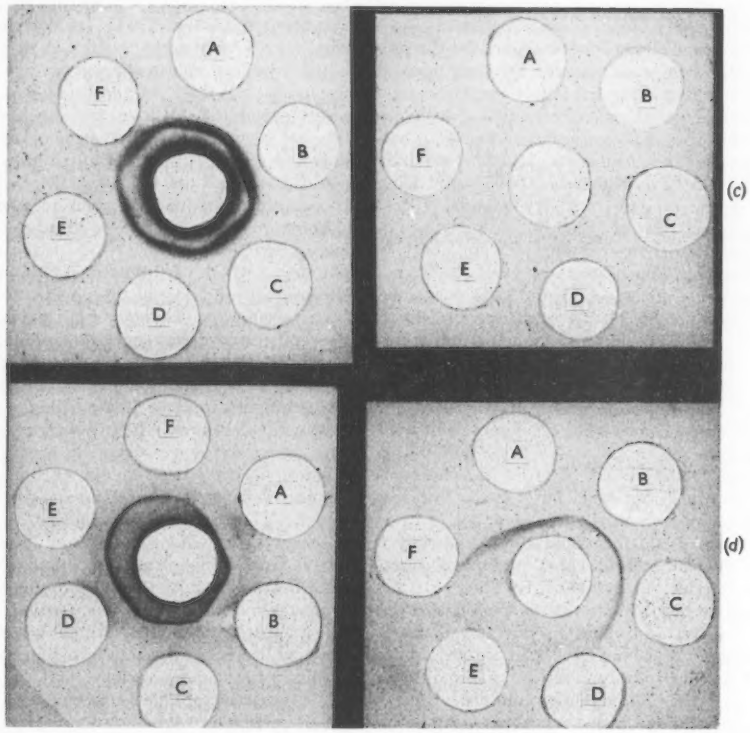

Fig. 3. Agar-gel precipitation reactions between extracts of gonadotrophin and antiserum to urinary DEAE 1. A–F are doubling dilutions of the antigens; the central hole contains the antiserum. (*a*) 0·1% of urinary DEAE 1; (*b*) 0·1% of pituitary-gland DEAE 1; (*c*) 0·1% of pituitary-gland CP 1; (*d*) 0·1% of pituitary-gland CP 3.

Table 11. *Titration of antisera and inhibition reaction*

+, Haemagglutination; −, no agglutination. In the titration 0·2 ml. of antiserum was mixed with 0·2 ml. of serum–sodium chloride solution and 0·05 ml. of red cells. With urinary DEAE 1 the end point was $\frac{1}{6400}$ and with pituitary-gland DEAE 1 the end point was $\frac{1}{1152}$. In the inhibition experiments a dilution of $\frac{1}{2}\frac{1}{3}\frac{1}{6}$ of the pituitary-gland antiserum was used and the agglutination was inhibited by dilution as far as $\frac{1}{128}$% of a urinary human menopausal gonadotrophin DEAE 1, and to $\frac{1}{2}$% of a benzoic acid–tungstic acid extract of human menopausal gonadotrophin. By biological assay the DEAE 1 fraction was 250 times as potent as the crude human menopausal gonadotrophin.

(i) Titration of antisera

Antiserum	Dilution of antiserum								
	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$	$\frac{1}{12800}$
Urinary DEAE 1	+	+	+	+	+	+	+	+	−
	$\frac{1}{144}$	$\frac{1}{288}$	$\frac{1}{576}$	$\frac{1}{1152}$	$\frac{1}{2304}$	$\frac{1}{4608}$			
Pituitary-gland DEAE 1	+	+	+	+	−	−			

(ii) Inhibition experiment with pituitary-gland DEAE 1 antiserum at a concn. of $\frac{1}{576}$

Gonadotrophin	Concn. of human menopausal gonadotrophin added (%, w/v)							
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$
HMG DEAE 1	−	−	−	−	−	−	−	+
	4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
HMG crude	−	−	−	−	+	+	+	+

DISCUSSION

Several methods have been described for the preparation of human gonadotrophin in highly purified form. Li, Squire & Gröschel (1960) employed zone electrophoresis on columns of ethanolized cellulose and column chromatography on diethylaminoethyl cellulose and Amberlite IRC 50 to purify follicle-stimulating and interstitial-cell-stimulating hormone from human pituitary glands. Ultracentrifugal analysis of the follicle-stimulating-hormone fraction showed remarkable similarity to our CP 1 fraction. The major component (70 %) had sedimentation coefficient 1·67 and CP 1 contained a major component (64 %) with sedimentation coefficient 1·64. The interstitial-cell-stimulating-hormone fraction of Li *et al.*, however, contained a major component (82 %) with sedimentation coefficient 2·4 differing considerably from our CM 2/2, which contained 95 % of a component with sedimentation coefficient 4·17. It is difficult to compare the biological activities because no comparison with the International Reference Preparation HMG 24 was reported by Li *et al.*

The initial steps of our method were adopted from the work of Steelman *et al.* (1959) and our observations are largely in agreement. Their follicle-stimulating-hormone fraction corresponds with our DEAE 1 fraction and their interstitial-cell-stimulating hormone with our CM 2/2. Comparisons of biological activity of products are again difficult owing to the lack of a common standard. Roos & Gemzell (1960) have prepared a pituitary-gland fraction rich in follicle-stimulating hormone which has been assayed against an international reference substance. They used chromatography on carboxymethyl- and diethylaminoethyl-cellulose followed by zone electrophoresis on a copolymerizate of polyvinyl chloride and polyvinyl acetate. The resulting follicle-stimulating-hormone fraction was tested by the augmentation method with rats instead of mice (Steelman & Pohley, 1953). It was assayed against HMG 20 A, a reference preparation similar in biological activity to HMG 24. The relative potency was 2040 times that of the standard, which compares well with 3002 of our CP 1.

We found a second fraction which was rich in follicle-stimulating hormone: this was CP 2. It was essentially homogeneous in the ultracentrifuge but had a higher sedimentation coefficient (3·07) than CP 1. It may be that the active principle in CP 1 was attached to a larger inert protein in CP 2 but its relationship to CP 1 is not yet clear.

The active material recovered in the three fractions CP 1, CP 2 and CM 2 accounted for over 50 % of the activity in the starting material HGH. 1/3. The only fraction containing detectable gonadotrophin, which was discarded, was left on the column of diethylaminoethylcellulose during the fractionation of CM 1. Its activity (per mg.) was very low compared with that of the other active fractions and in quantity it did not account for more than 0·5 % of the original material. The fractionation of CM 2 on carboxymethylcellulose was not very efficient. No active material could be detected on the column although only about 20 % could be found in CM 2/2. Other methods are being investigated for the fractionation of CM 2.

Fractions rich in follicle-stimulating hormone have been obtained from postmenopausal urine by the method of Johnsen (1955), followed by chromatography on calcium phosphate. It has not been possible to reach such high potencies as with the pituitary-gland gonadotrophins, the best pituitary-gland fractions having a potency about 10 times that of the best urinary preparation with the augmentation assay. Bourillon, Got & Marcy (1960) have obtained a highly active gonadotrophin from postmenopausal urine by precipitation with ethanol, adsorption on kaolin, chromatography on ion-exchange materials and zone electrophoresis on starch. The product was active in the mouse uterine-weight assay at a dose of 1 μg. It was not assayed directly against the standard, but other information given by the authors suggests that this represents a relative potency of about 1000 in terms of the standard, which implies that its potency lies between those of our best urinary and pituitary-gland preparations.

The gonadotrophins from both urine and from pituitary glands have shown remarkable stability. They have been stored in 0·9 % sodium chloride solution at $-15°$ for over 12 months without detectable loss of activity. During this time they have been removed and thawed at frequent intervals for examination.

The production of antibodies to these gonadotrophins has been carried out by a new technique. They were adsorbed strongly by bentonite, which was given to rabbits as a suspension. A powerful antibody was detected after the administration of 3 mg. of pituitary-gland DEAE 1 and it has been found that on immunizing the rabbit again after a rest of 9 months only 0·9 mg. of the same antigen was required to restore the titre to its original value. A statistical comparison of the new technique with other established techniques has not been made because of the limited supplies available, but the method of alum suspension has been ineffective in our hands. This is probably because the gonadotrophin remains in solution. Antibodies have also been produced with Freund's adjuvant (Butt, Crooke, Cunningham & Gell, 1960), but large quantities of antigen were required.

Antibodies to both urinary and pituitary-gland antigens were effective *in vivo* in inhibiting the

activity of gonadotrophins from urine or pituitary glands in the mouse but they were ineffective against human chorionic gonadotrophin in the rat. A continuation of our previous studies (Butt, Crooke & Cunningham, 1960) has confirmed that the antibody to human chorionic gonadotrophin is effective not only against human chorionic gonadotrophin but also against human menopausal gonadotrophin and pituitary-gland gonadotrophin.

It has not been possible to use immuno-electrophoresis in this study since no precipitation can be observed in agar gel. The red-cell-haemagglutination technique, however, gives a satisfactory end point. We have used the method of Ling (1960) for preparing the red cells since it was found that the sensitized cells were much more stable than those prepared by the tannic acid method. Some have been used satisfactorily 2 months after they were coated. Since inhibition of the haemagglutination reaction was obtained with microgram quantities of pituitary-gland or chorionic gonadotrophin the technique may be useful for the quantitative estimation of gonadotrophins in urine or serum. In view of the cross-reactions of pituitary-gland and chorionic gonadotrophins, however, the results will have to be interpreted with care.

SUMMARY

1. Gonadotrophins were prepared from human urine and pituitary glands and purified by chromatography on carboxymethyl- and diethylaminoethyl-cellulose and calcium phosphate.

2. Two fractions, one with mainly follicle-stimulating-hormone activity and one with mainly interstitial-cell-stimulating-hormone activity, were obtained.

3. The first follicle-stimulating-hormone fraction was 3002 times as potent as the International Standard Preparation HMG 24 by the ovarian-augmentation assay. It showed two components in the ultracentrifuge, the major one (64%) having sedimentation coefficient 1·64. The second follicle-stimulating-hormone fraction was 1014 times as potent as the standard and was homogeneous in the ultracentrifuge with sedimentation coefficient 3·07.

4. The best urinary fractions had about one-tenth of the activity of the best pituitary-gland fractions.

5. Antibodies were raised to some of the fractions from urine and pituitary glands and when tested *in vivo* they were found to inhibit both urinary human menopausal gonadotrophin and pituitary-gland gonadotrophin but were ineffective against human chorionic gonadotrophin. They failed to produce precipitin lines by the agar-gel-diffusion technique but could be used in the red-cell-haemagglutination technique.

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Analytical Separations by High-Voltage Paper Electrophoresis

AMINO ACIDS IN PROTEIN HYDROLYSATES

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The determination of the amino acid composition of peptides and proteins is usually carried out on acid hydrolysates by ion-exchange chromatography, by following the methods developed by Moore & Stein and their collaborators (Moore, Spackman & Stein, 1958). This method is precise but time-consuming, requiring up to 600 individual determinations for a single protein analysis. Automatic scanning of the column effluent (Spackman, Stein & Moore, 1958) obviates this objection, but increases complication and cost.

An alternative group of methods for amino acid analysis are based on paper-chromatographic separation of the individual amino acids, followed by quantitative determination by means of some modification of the ninhydrin reaction. A considerable literature (reviewed by Lederer & Lederer, 1957; Hanes, 1961) describes the many variants of this method, but most are subject to considerable errors due mainly to the limitations of the ninhydrin reaction, and to losses on the paper during the chromatographic separation and the subsequent manipulations. An exhaustive study of these sources of error has been made by Hanes and his associates (Connell, Dixon & Hanes, 1955; Hanes, 1961; Wade, Matheson & Hanes, 1961; Hanes, Harris, Moscarello & Tigane, 1961; Matheson, Tigane & Hanes, 1961; Tigane, Wade, Tze-Fei Wong & Hanes, 1961), who have developed two new paper-chromatographic systems for the separation of 16 of the 18 common amino acids, and also a modified ninhydrin reagent. These improvements constitute a considerable advance over previous methods, but at the cost of a relatively complex technique. Recoveries of individual amino acids were within $\pm 10\%$. No applications to protein hydrolysates were reported.

Levy (1954) has described a method of amino acid analysis based on the paper-chromatographic separation of the dinitrophenyl derivatives, followed by spectrophotometric determination at 360 mμ. This method appears to be markedly inferior to ion-exchange chromatography (see Hedbom, 1961, for a direct comparison). Whitehead (1958) has described an isotope-dilution method based on acetylation of the amino acids with [^{14}C]- and [^{3}H]-acetic anhydride, and a combined chromatographic separation of the acetyl compounds. This method does not appear to have been widely used, although it is applicable to microgram amounts of protein.

Recent advances in the technique of high-voltage paper electrophoresis (reviewed comprehensively by Michl, 1958) have provided an analytical method of extremely high resolving power for small molecules. The present paper describes systems for the complete separation of all the amino acids commonly occurring in protein hydrolysates. Combined with the improved cadmium–ninhydrin method of Heilmann, Barollier & Watzke (1957) this provides a new method of amino acid analysis, which reduces the estimations required to one for each amino acid present in the hydrolysate, while the precision is comparable with that obtained with the ion-exchange method; 250 μg. of protein suffices for a complete amino acid analysis. Röwe, Ferber & Fischer (1958) have described an analogous method, which was, however, applicable to only 10 of the 18 commonly occurring amino acids. Preliminary accounts of the present work have already been published (Atfield & Morris, 1960*a*, *b*).

EXPERIMENTAL

Apparatus

The apparatus used for the electrophoretic separation is based on Gross's (1956, 1961) modification of Michl's (1953) design, in which the paper strip is held horizontally between two cooled surfaces. The apparatus is illustrated in Fig. 1. It consists essentially of two anodized aluminium–copper alloy plates, *AA*, 105 cm. × 15 cm. × 2·5 cm., milled to a surface flatness tolerance of 0·001 in. The plates are provided with internal channels, through which aqueous 20% methanol maintained at $2 \pm 1°$ by a thermostated refrigeration unit is circulated at approx. 100 ml./sec. to provide cooling.

The paper electrophoresis strip *C* is insulated electrically from the cooling plates *AA* by the 0·014 in. thick polythene films *BB* (Telegraph Construction and Maintenance Co. Ltd., Farnborough, Kent), which are 5 cm. wider than the cooling plates, to provide adequate insulation under conditions of high humidity. The electrophoresis strip is connected to the electrode vessels by bridges *DD*, which consist of two layers of moist Whatman 3MM filter paper enclosed in cellophan (grade 300, uncoated). The heavy

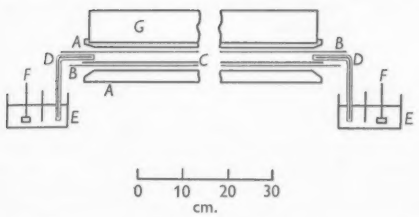

Fig. 1. Schematic view of high-voltage electrophoresis apparatus. *AA*, Cooling plates; *BB*, polythene insulating films; *C*, paper electrophoresis strip; *DD*, cellophan-covered bridges; *EE*, electrode vessels; *FF*, platinum electrodes; *G*, pneumatic device (see text).

polythene electrode vessels *EE* have a labyrinth arrangement to minimize diffusion of electrolysis products towards the electrophoresis strip. The electrodes *FF* are platinum plates (2 cm. × 0·5 cm.).

During operation the cooling plates and insulating films are maintained in close contact with the electrophoresis strip by the pneumatic device *G*, which contains a rubber bag inflated to 0·14 kg./cm.². *G* is attached to the bottom plate by metal straps (not shown), so that a uniform pressure is exerted on the free upper cooling plate. It is essential to maintain the surfaces of the cooling plates in good condition for efficient removal of heat. In practice outputs up to 500 w could be dissipated over 8 hr. periods without evidence of excessive heating.

The potential gradient for electrophoresis is provided by a transformer–selenium rectifier unit giving outputs up to 100 ma at 12 000 v. Variation of the applied potential is obtained by switch selection of multiple secondary tappings, and the output is smoothed by a capacitance–choke–capacitance filter circuit. The entire apparatus is enclosed in a Perspex cover, removal of which interrupts the primary circuit of the power unit.

Materials

Whatman 3 MM filter paper has been used as the stabilizing medium throughout this work. Several other grades examined gave inferior resolution.

Preparation and hydrolysis of performic acid-oxidized insulin. The method used is based on that of Hirs (1956). The insulin hydrochloride used was a three times recrystallized specimen (Boots Pure Drug Co. Ltd.). Since Thompson (1954) has shown that performic acid oxidation of tyrosine or tyrosine-containing proteins in the presence of HCl leads to the formation of chlorotyrosine, it was necessary to remove chloride ions before oxidation.

Insulin (15 mg.) was dissolved in conc. NH_3 solution (sp.gr. 0·88; 50 μl.) and water (3 ml.) added. Dowex 2 X 8 (acetate) ion-exchange resin (50 mg.) was added to the insulin solution, and the mixture shaken for 30 min. The ion-exchange resin was removed by filtration and washed with water, and the combined filtrate and washings were freeze-dried. Performic acid was prepared by adding H_2O_2 (30%, v/v; 0·5 ml.) to formic acid (A.R. grade 98–100%; 9·5 ml.), and keeping the mixture for 2 hr. before use. Freeze-dried chloride-free insulin (14 mg.) was dissolved in formic acid (98–100%; 0·5 ml.) and anhydrous methanol (0·1 ml.) added with stirring. The insulin solution and the performic acid solution were cooled to 0°, and 1 ml. of the performic acid solution was added to the insulin solution. The reaction was allowed to proceed for 5 hr. at 0° and was terminated by rinsing the contents of the reaction flask into ice-cold water to a total volume of 40 ml. The diluted solution was frozen immediately and the solvents were removed by freeze-drying. The residue was dissolved in water (4 ml.), transferred to a small flask with more water (4 ml.) and again freeze-dried. The product was further dried over P_2O_5 *in vacuo* for analysis.

Hydrolysis was carried out on a 5 mg. sample in a sealed tube with constant-boiling-point HCl (0·1 ml.) for 24 hr. at 110°. The hydrolysate was repeatedly evaporated to dryness with small volumes of water to remove excess of HCl. The final product was dissolved in aqueous 10% (v/v) propan-2-ol (0·1 ml.) for transfer to the electrophoresis strip.

Electrolyte solutions. The compositions given are critical and should be strictly adhered to for best results: (*a*) Pyridine–acetic acid–water, pH 5·2; pyridine (A.R. grade; redistilled; 20·0 g.), acetic acid (A.R. grade; 99·6%; 9·5 g.) and water to 1 l. (*b*) Formic acid–acetic acid–water, pH 1·85; acetic acid (A.R. grade; 99·6%; 78·0), formic acid (A.R. grade; 98–100%; 25·0 g.) and water to 1 l. (*c*) Formic acid–acetic acid–cadmium acetate–water, pH 1·81; acetic acid (A.R. grade; 99·6%; 200·0 g.), formic acid (A.R. grade; 98–100%; 20·0 g.), cadmium acetate (A.R. grade; 1·06 g.) and water to 1 l.

Cadmium–ninhydrin reagent. Cadmium acetate (A.R. grade; 0·050 g.) was dissolved in a mixture of water (5·0 ml.) and acetic acid (1·0 ml.). Acetone (50 ml.) was added and the mixture shaken until any precipitate had redissolved. Ninhydrin (A.R. grade; 0·500 g.) was dissolved in the mixture. The reagent was prepared fresh daily.

Cadmium–isatin reagent. Cadmium acetate (A.R. grade; 0·25 g.) was dissolved in water (1·25 ml.), and acetic acid (A.R. grade; 0·25 ml.) and propan-2-ol (23·75 ml.) were added to the solution. Isatin (0·25 g.) was dissolved in the mixture. The reagent was prepared fresh daily.

Procedure

A strip of Whatman 3 MM filter paper (120 cm. × 13·5 cm.) was marked with a light transverse pencil line 14·5 cm. from one end and immersed in the appropriate electrolyte solution until saturated. It was then drawn through a twin rubber-roller 'mangle' to remove excess of liquid. The upper roller was spring-loaded with adjustable pressure. It was set to give a moderately dry paper (95–100 g. of electrolyte/100 g. of dry paper). Satisfactory results can also be obtained by blotting with dry filter paper, although reproducibility is then more difficult to achieve. The paper strip was laid on a glass plate with paper strips (14 cm. × 2 cm.) laid transversely above and below the electrophoresis strip in the vicinity of the starting line. The whole was covered with a 0·014 in.-thick polythene film, and left for 10 min. to equilibrate. The bridge strips were prepared by immersing a strip (30 cm. × 13·5 cm.) of Whatman 3 MM filter paper in electrolyte to saturation, doubling it to form a pad (15 cm. × 13·5 cm.), roller-pressing to remove excess of liquid, and wrapping the pad in cellophan sheet (grade 300) that had been previously soaked in the electrolyte for at least 12 hr. After 10 min. the paper drying strips were

removed from the electrophoresis strip and the latter was supported on a slotted polythene bridge piece, so that the starting line, although supported, was not in direct contact with a solid surface.

The hydrolysate solution (5 μl.) was then applied to the paper from a micropipette to give a uniform line approximately 11 cm. long. The application is facilitated by the addition of an anionic dye such as acid fuchsin or neucoccin to the test mixture. The regularity of application can then be checked visually, while the dye migrates anodically away from the amino acid mixture on application of the electric potential. A length (2·5 cm.) of the strip at the anodic (starting) end was cut off and transferred to a weighing bottle for moisture determination. The polythene film covering the electrophoresis strip was then inverted and the strip laid on it before transference to the lower cooling plate of the electrophoresis apparatus. A portion (2·5 cm.) at each end of the electrophoresis strip was folded over to form contact pads for the bridge strips, which were placed in a position overlapping the electrophoresis strip by approximately 2 cm., and the whole was covered by another polythene film. The upper cooling plate was lowered and secured, and the pneumatic pressure bag inflated to 0·14 kg./cm.^{2}. Good contact between the bridge strips and the electrophoresis strip was ensured by insertion of small rolls of polythene film into the end recesses in the cooling plates. The electrode vessels were filled with electrolyte solution, the cover was put in place, and the potential gradient applied. During operation the electric current tends to increase and it should be checked and if necessary adjusted at 15 min. intervals. A constant current supply would undoubtedly be superior, and the design of a suitable power supply is being currently investigated.

At the end of the separation, the potential gradient was shut off, the air pressure released, the paper strip transferred to a horizontal glass plate, and most of the liquid removed by infrared heating in an air stream for 10 min. The strip was finally dried for 15 min. at 80°. The two-stage drying procedure prevents deterioration of the separated zones by liquid movement within the strip. The dried strip was dipped in the cadmium–ninhydrin reagent, and stored for 20 hr. in a dark, all-glass, air-tight vessel, provided with a dish containing conc. H_2SO_4 to maintain a NH_3-free atmosphere. Quantities (5, 10 and 20 μg.) of all the amino acids investigated were transferred in 0·1 ml. volumes to strips of Whatman 3MM paper that had been wetted with the appropriate electrolyte solution and roller-pressed free from excess of liquid. These were dried, dipped in ninhydrin reagent and developed together with the electrophoresis strip. Next day the bright-red zones were cut out together with appropriate blank control strips, cut into pieces (approx. 1 cm. × 0·5 cm.), and transferred to individual glass-stoppered test tubes (12 cm. × 1·6 cm.). Redistilled absolute methanol (8·00 ml.) was added to each, and the tubes were stoppered and secured and rotated end-for-end in a rotating frame-shaking machine for 30 min. The clear red solutions were freed of paper fibres by pressure filtration through cylindrical funnels provided with sintered-glass filter disks (porosity 2). The extinction was measured at 500 mμ for the amino acids, and at 352 mμ for the yellow reaction product given by proline, by using 1 or 2 cm. cells. The amino acid concentration–extinction graphs showed satisfactory linearity up to an extinction of 0·9 (Atfield & Morris, 1960*b*), and the individual colour yields remained within ±3·5% over a period of 12 months.

If the proline content is low (as in insulin), the decreased sensitivity of the ninhydrin reaction may necessitate an alternative procedure based on the colorimetric method of Hrabětová & Tupý (1960). The position of the proline zone was located in the formic acid–acetic acid–cadmium acetate electrophoresis by means of markers on the edges of the strip. The corresponding area of paper was cut out, dried, dipped in the cadmium–isatin reagent and heated for 30 min. at 80°. Proline gives a green zone on a yellow background under these conditions. The strip was washed repeatedly with water at 30° until the background was white or very pale yellow, and the proline zone was deep blue. The paper containing the blue band was cut into small pieces, transferred to a glass-stoppered test tube (12 cm. × 1·6 cm.) and eluted with water-saturated phenol (5·00 ml.) by end-over-end shaking for 15 min. The blue eluate was filtered through a sintered-glass funnel under positive pressure and the extinction measured at 610 mμ. A control prepared from an adjacent area of the electrophoresis strip, and proline standards (5 and 10 μg.), were prepared in a similar manner. In agreement with the findings of Heilmann *et al.* (1957) and Hrabětová & Tupý (1960), satisfactory agreement with the Beer–Lambert Law was found for both colorimetric methods up to extinctions of approx. 0·6.

RESULTS

Three electrophoretic separations in different electrolyte solutions are necessary to separate the eighteen amino acids commonly found in protein hydrolysates. These separations are shown in Fig. 2.

The first electrophoresis carried out in pyridine–acetate buffer at pH 5·2 for 200 min. at 75 v/cm. separates the basic amino acids, lysine, histidine and arginine (and also hydroxylysine and ornithine if present), as cations (Fig. 2*a*) and the acidic amino acids, cysteic, aspartic and glutamic acid, as anions (Fig. 2*b*). The neutral amino acids form an unresolved zone that is displaced 1·5–2 cm. towards the cathode by electro-osmotic flow through the paper.

The second electrophoresis is carried out in a 2·5% formic acid–7·8% acetic acid medium at pH 1·85 and gives a good resolution of the majority of the neutral amino acids. The resolved amino acids (in order of mobilities) are glycine, alanine, serine, valine, leucine + isoleucine (partially resolved), threonine, proline (yellow ninhydrin reaction), glutamic acid, phenylalanine + aspartic acid (not resolved), methionine sulphone and tyrosine. Tryptophan, if present, as in an enzymic digest, migrates more slowly than tyrosine. A good separation is obtained in 270 min. at 100 v/cm. (Fig. 2*c*).

A third electrophoresis is necessary to resolve those amino acids that are not resolved in the formic acid–acetic acid medium. This is carried out

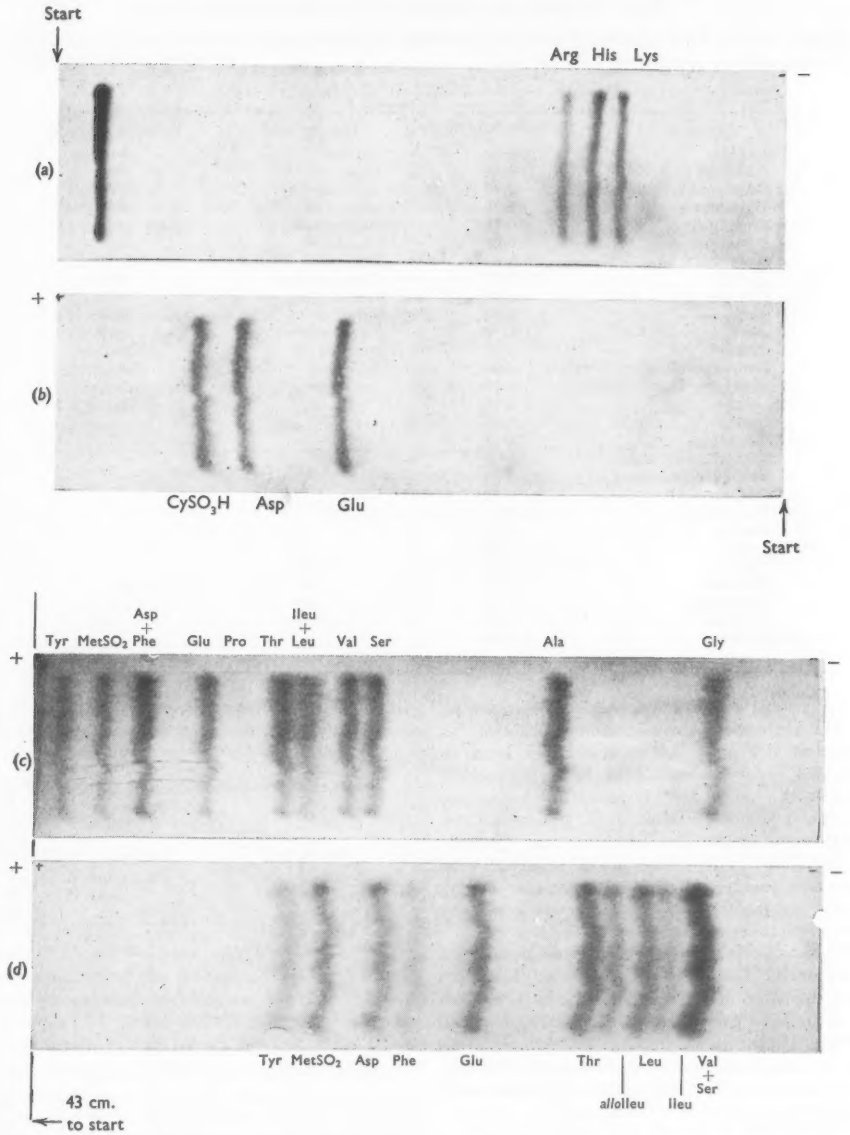

Fig. 2. Separation of amino acids by high-voltage paper electrophoresis. (*a*) Pyridine–acetate buffer, pH 5·2. From the left unresolved neutral amino acids, arginine, histidine and lysine. (*b*) Pyridine–acetate buffer, pH 5·2. Cysteic acid, aspartic acid and glutamic acid. (*c*) Formic acid–acetic acid, pH 1·85. Tyrosine, methionine sulphone, phenylalanine + aspartic acid (unresolved), glutamic acid, threonine, leucine + isoleucine (unresolved), valine, serine, alanine and glycine. (*d*) Formic acid–acetic acid–cadmium acetate, pH 1·81. Tyrosine, methionine sulphone, aspartic acid, phenylalanine, glutamic acid, threonine, *allo*isoleucine, leucine, isoleucine and valine + serine (unresolved).

Table 1. *Mobilities of amino acids relative to alanine*

System *A*: 2·5% formic acid–7·8% acetic acid, pH 1·85, 100 v/cm. System *B*: 2·0% formic acid–20% acetic acid–4 mM-cadmium acetate, pH 1·81, 100 v/cm.

A		*B*	
Amino acid	Relative mobility	Amino acid	Relative mobility
Alanine	1·00	Alanine	1·00
β-Alanine	1·45	Aspartic acid	0·59
α-Aminobutyric acid	0·90	Glutamic acid	0·66
Arginine	1·31	Isoleucine	0·80
Asparagine	0·71	*allo*Isoleucine	0·76
Aspartic acid	0·61	Leucine	0·78
Cysteine	0·60	Methionine sulphone	0·55
Cystine	0·59	Phenylalanine	0·61
Glutamic acid	0·67	Proline	0·69
Glutamine	0·69	Serine	0·81
Glycine	1·14	Threonine	0·73
Histidine	1·31	Tyrosine	0·53
Hydroxyproline	0·54	Valine	0·81
Isoleucine	0·77		
Leucine	0·77		
Lysine	1·47		
Methionine	0·71		
Methionine sulphone	0·57		
Methionine sulphoxide	0·60		
Ornithine	1·52		
Phenylalanine	0·61		
Proline	0·69		
Serine	0·83		
Taurine	0·03		
Threonine	0·75		
Tryptophan	0·46		
Tyrosine	0·53		
Valine	0·81		

in a 2% formic acid–20% acetic acid solution at pH 1·81, containing 4 mM-cadmium acetate, for 420 min. at 100 v/cm. The separation sequence in descending order of mobilities is valine + serine (unresolved), isoleucine, leucine, *allo*isoleucine, threonine, proline, glutamic acid, phenylalanine, aspartic acid, methionine sulphone and tyrosine (Fig. 2*d*). The basic amino acids, together with glycine and alanine, migrate off the paper during the increased duration of electrophoresis necessary for the separation of the isomeric leucines.

Owing to the controlled and constant wetness of the stabilizing medium, measured mobilities have shown less than 2% variation over a period of 18 months, and over migration distances from 40 to 80 cm. They provide unequivocal identification of all the amino acids studied. The relative mobilities of a number of naturally occurring amino acids and amines are given in Tables 1 and 2.

Quantitative analyses

Analyses of synthetic mixtures containing approximately 10 and 20 μg. of 17 amino acids are given in Tables 3 and 4. Glutamic acid can be estimated in all three systems and provides an internal intercomparison standard, and several

Table 2. *Mobilities of amino acids relative to glutamic acid (acidic amino acids) and arginine (basic amino acids)*

System was pyridine–acetic acid, pH 5·2, 70 v/cm.

	Mobility relative to	
Amino acid	Arginine	Glutamic acid
Arginine	1	—
Aspartic acid	—	1·21
Cysteic acid	—	1·30
Glutamic acid	—	1
Histidine	1·06	—
Hydroxylysine	1·04	—
Lysine	1·11	—

other amino acids can be measured in both the formic acid–acetic acid and cadmium–formic acid–acetic acid systems. Isoleucine presents a special problem, as only one preparation of five examined was free of the *allo*-isomer and other ninhydrin-reactive impurities. The low recoveries of this amino acid in Tables 3 and 4 reflect this uncertainty.

Triplicate analyses of the hydrolysates of two samples of performic acid-oxidized insulin prepared as described in the Experimental section are

Table 3. *Recoveries from approximately* 10 *μg. quantities of amino acids*

F–A, Cd–F–A and P–A refer to the formic acid–acetic acid, cadmium–formic acid–acetic acid and pyridine–acetic acid media respectively.

Amino acid	Present (μg.)	Found (μg.) F–A	Cd–F–A	P–A	Error (%)
Alanine	9·1	9·1	—	—	±0
Arginine	9·4	—	—	9·2	−2·1
Aspartic acid	9·4	—	—	9·1	−3·2
Cysteic acid	14·1	—	—	14·3	+1·1
Glycine	9·5	9·6	—	—	+1·05
Glutamic acid	9·0	9·1	8·7	8·95	−3·2 (mean)
Histidine	9·0	—	—	9·0	±0
Isoleucine	9·2	—	8·4	—	−8·6
Leucine	9·3	—	9·2	—	−1·1
Lysine	12·1	—	—	12·1	±0
Methionine sulphone	6·4	6·5	6·2	—	−0·8 (mean)
Phenylalanine	9·8	—	10·1	—	+3·1
Proline	10·3	10·25	—	—	−0·8
Serine	9·2	9·2	—	—	±0
Threonine	9·9	9·8	—	—	−1·0
Tyrosine	9·7	—	9·9	—	+2·1
Valine	12·0	12·0	—	—	±0

Table 4. *Recoveries from approximately* 20 *μg. quantities of amino acids*

For abbreviations see Table 3.

Amino acid	Present (μg.)	Found (μg.) F–A	Cd–F–A	P–A	Error (%)
Alanine	20·0	20·05	—	—	+0·25
Arginine	17·15	—	—	17·6	+2·6
Aspartic acid	26·0	—	27·0	25·2	+0·4 (mean)
Cysteic acid	22·0	—	—	21·9	−0·45
Glycine	20·4	20·0	—	—	−1·95
Glutamic acid	21·9	21·7	22·2	21·5	−0·45 (mean)
Histidine	16·3	—	—	16·3	±0
Isoleucine	18·55	—	17·3	—	−6·7
Leucine	18·0	—	17·7	—	−1·7
Lysine	16·35	—	—	16·1	−1·5
Methionine sulphone	19·8	20·3	20·5	—	+2·0 (mean)
Phenylalanine	19·75	—	18·8	—	−4·8
Proline	23·05	23·2	—	—	+0·65
Serine	19·5	19·7	—	—	+1·0
Threonine	20·7	20·95	19·4	—	−2·4 (mean)
Tyrosine	16·9	16·7	—	—	−1·2
Valine	20·95	20·5	—	—	−2·15

given in Table 5, together with the calculated composition based on the known structure of insulin. The serine and threonine values have been corrected for loss on hydrolysis, and all values are corrected for the moisture and ash content of the oxidized insulin. The proline values were obtained by the isatin method.

The recoveries from synthetic mixtures were within ± 5 %, with the exception of the isoleucine values, for reasons noted above. The analyses of performic acid-oxidized insulin, although in satisfactory internal agreement (all within ± 10 %, and the majority within ± 5 %), are in less satisfactory agreement with the calculated values, although the majority are within ± 10 %. This may reflect the purity of the amino acid standards (for example histidine and proline) or the uncertainty in the composition of the actual insulin preparations used. The improved results of the second analysis suggest that an accuracy of ± 10 % is within the range of the method.

DISCUSSION

The mobility u of an ion of net charge q, and diffusion coefficient D, is given by the expression:

$$u = \frac{qD}{kT}, \qquad (1)$$

where T is the absolute temperature, and k is the Boltzmann constant. Equation (1) is independent

Table 5. *Amino acid analyses of two samples of performic acid-oxidized insulin*

Values are given as g. of amino acid/100 g. of oxidized insulin.

Amino acid	Experimental I			Experimental II			Calculated
Alanine	4·4	4·4	3·6	3·9	4·0	4·0	4·4
Arginine	2·3	2·3	2·3	2·8	2·8	2·8	2·9
Aspartic acid	6·0	5·9	6·0	6·6	6·6	6·6	6·6
Cysteic acid	14·75	15·35	15·6	15·9	16·5	15·6	16·8
Glutamic acid	16·3	16·4	16·6	16·1	16·1	16·2	17·1
Glycine	5·25	4·75	5·5	5·1	4·95	5·3	5·0
Histidine	4·4	4·55	4·5	4·4	4·65	4·6	5·2
Isoleucine	2·1	2·1	2·1	1·7	1·8	1·8	2·2
Leucine	13·0	13·1	13·1	12·1	12·4	13·8	13·1
Lysine	1·8	1·9	2·1	2·3	2·4	2·1	2·4
Phenylalanine	10·8	10·8	11·2	8·8	9·2	8·7	8·2
Proline	1·6	1·7	1·9	1·6	1·8	1·6	1·9
Serine	4·5	4·5	4·75	3·5	3·6	3·7	5·2
Threonine	1·9	1·9	1·9	1·9	1·9	2·0	2·0
Tyrosine	11·9	11·5	11·9	12·0	11·9	12·0	12·0
Valine	8·0	8·2	9·1	8·8	8·8	9·0	9·7
Total	109·0	109·35	112·15	107·5	109·4	109·8	114·7

Table 6. *Experimental and calculated relative mobilities of amino acid cations in formic acid–acetic acid solution*

Amino acid	u (exp.)	u (calc.)		
pH	1·85	2·1	2·0	1·9
Histidine	1·31	1·32	1·37	1·42
Glycine	1·14	1·20	1·19	1·19
Alanine	1·00	1·00	1·00	1·00
α-Aminobutyric acid	0·90	0·88	0·87	0·86
Serine	0·82	0·86	0·88	0·89
Valine	0·81	0·81	0·81	0·81
Leucine	0·77	0·79	0·78	0·78
Threonine	0·75	0·76	0·78	0·79
Methionine	0·72	0·75	0·76	0·76
Proline	0·69	0·67	0·70	0·73
Glutamic acid	0·67	0·68	0·70	0·71
Aspartic acid	0·61	0·58	0·54	0·52
Phenylalanine	0·61	0·89	0·87	0·85
Tyrosine	0·53	0·63	0·64	0·65
Tryptophan	0·46	0·73	0·72	0·72

of the shape and size of the ion, but is only valid in media of low ionic strength in which interionic interactions may be neglected. This may be assumed to be approximately true for the formic acid–acetic acid medium used in this work, where the major ionic species will be protons at approximately 0·01 molarity. Absolute mobilities cannot be determined by paper electrophoresis for a variety of reasons (discussed by Svensson, 1956), but mobilities relative to that of a chosen ion can be measured with high precision, provided that the ionic migration is linear both in time and in applied potential gradient. These conditions rarely obtain in conventional paper electrophoresis, owing to the evaporation of solvent from the electrophoresis strip and the consequent inward flow of buffer from the electrode vessels. This is not the case in the present method, as the use of cellophan-enclosed bridges (Weber, 1951; Michl, 1953) prevents hydrodynamic flows within the paper, and adequate cooling minimizes evaporation. In actual practice the moisture content of the strip changes by less than 5 % during operation for 8 hr. at 300 w dissipation. This constancy of paper wetness is reflected in linearity of ionic migration both in time and in applied potential. Measurements of the distance of migration of the alanine cation in the formic acid–acetic acid system as a function of time at constant current (applied potential) and as a function of current for a constant period of migration, show that the linearity of migration (within 1 % in both cases) justifies the measurement of relative mobilities up to distances of at least 80 cm.

For two ionic species A and B, equation (1) may be written in the form:

$$\frac{u_A}{u_B} = \frac{q_A D_A}{q_B D_B}, \qquad (2)$$

where the subscripts identify the species. Table 6 shows the mobilities of several amino acid cations measured relative to the mobility of alanine in the formic acid–acetic acid system at pH 1·85 and 4°. The Table also shows the relative mobilities calculated from equation (2) from values of the net charges obtained at three pH values from the Henderson–Hasselbalch equation, without correction for thermodynamic activities, and values of the diffusion coefficients at 1° obtained either from Longsworth's (1952) measurements, or calculated

from the molecular weights by means of his empirical equation. Several points emerge from this comparison:

(1) The agreement is in general good enough (within 5%) to justify application of equation (2) to the prediction of relative mobilities in this system.

(2) The aromatic amino acids phenylalanine, tyrosine and tryptophan migrate more slowly than predicted. This is undoubtedly due to reversible adsorption on the paper. This phenomenon is already well established for these substances, and tryptophan can in fact be separated from other amino acids by paper chromatography in aqueous solutions (e.g. Dalgleish, 1956).

(3) The calculated values for pH 2·1 agree better with the experimental values than those calculated for pH 1·9, nearest to the actual pH of the electrophoresis medium. This is particularly evident for threonine, which should migrate faster than leucine at pH 1·85, whereas in fact it migrates more slowly in agreement with the predicted behaviour at pH 2·1. This anomaly can be explained by the suggestion of Davies (1949) that the H^+ ion potential within a porous solid phase will differ from that in free solution by the potential difference (mainly electrokinetic) through which a proton must pass in order to reach the interior of the porous solid. Thus if pH_i is the pH inside the porous phase and pH_o is the pH in free solution in equilibrium with the solid phase:

$$pH_i - pH_o = \frac{V}{0{\cdot}059}, \qquad (3)$$

where V is the electrokinetic potential. The value of V as estimated from electro-osmotic flow in the formic acid–acetic acid system is small and positive, so that the direction of the observed anomaly is predicted correctly by equation (3), and its magnitude, 0·25 pH unit, would require the reasonable value of 15 mv for V. The neglect of activity coefficients in the calculation of q may also affect the values of u although this may be expected to balance out in the relative values.

The present method appears to be capable of separating completely ions differing in mobility by about 1%. This degree of resolution is a result of the linearity of migration in time, and there appears to be no reason why the migration path should not be extended to provide further resolving power. A 2·5 m. machine in construction in this Laboratory may enable the majority of the amino acids to be separated in a single electrophoresis.

The use of Cd^{2+} ions in the third electrophoresis medium presents some interesting features as this and certain other heavy-metal ions produce a small but definite specific retardation of the dicarboxylic and hydroxy amino acids relative to the aliphatic or aromatic amino acids (Table 1). This effect has been exploited in the present work to separate aspartic acid from phenylalanine, and it also accounts for the decreased resolution of serine and valine in the cadmium-containing medium. Although only a small proportion of the amino acid would be expected to form complexes with the metal at pH 1·81 (Gurd & Wilcox, 1956), this may nevertheless be sufficient to account for the retardation, and it suggests that paper electrophoresis under carefully controlled conditions may be a useful method for the investigation of complex formation.

The analytical results on performic acid-oxidized insulin provide evidence that the method is a practical one for the amino acid analysis of proteins on the milligram scale. Several modifications of the ninhydrin method for the colorimetric determination of the amino acids were examined in the course of this work, but the cadmium–ninhydrin procedure of Heilmann *et al.* (1957) was clearly superior as regards low blanks, linearity of colour response over a wide concentration range and reproducibility.

SUMMARY

1. An apparatus and experimental details for high-voltage paper electrophoresis under conditions of constant temperature and paper wetness are described.

2. The application of the method to the separation of the amino acids commonly occurring in protein hydrolysates is described. Eighteen amino acids can be separated completely, by three electrophoretic experiments at pH 5·2, 1·85 and 1·81, at potential gradients of 70–100 v/cm.

3. The mobilities of several amino acid cations relative to alanine are tabulated, and a method for calculation of these mobilities from the net charge and diffusion constant is described and compared with experiment.

4. A method of quantitative determination of the resolved amino acid zones by use of the cadmium–ninhydrin reaction is described, and analyses of synthetic amino acid mixtures and replicate analyses of hydrolysates of performic acid-oxidized insulin are given to illustrate the utility of the method.

We are indebted to Dr D. Gross for details of construction of his electrophoresis apparatus, and to Professor F. Weygand, Munich, for a gift of isoleucine free from the *allo*-isomer.

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An Apparatus for the Automatic Measurement of Oxygen Uptake by Electrolytic Replacement of the Oxygen Consumed

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In studies of the respiration of plant tissue, particularly in relation to terminal oxidases, measurement of the rate of uptake of oxygen from an atmosphere of controlled oxygen pressure is necessary. For short periods of time and small amounts of tissue a Warburg type of apparatus is suitable but in studies of intact tissue, where whole organs must be used (0·5–200 g.) and where the duration of an experiment is prolonged, an apparatus that maintains the partial pressure of oxygen constant while giving a continuous record of uptake is needed. The apparatus to be described replaces the oxygen consumed by the tissue by oxygen generated electrolytically, and automatically records the volume added. This principle of electrolytic replacement of oxygen has been used before (Fernandes, 1923; Wetzel, 1927; Bennet-Clark, 1932; Ulrich, 1940), but the accuracy of the estimations has been greatly increased by the use of a new type of electrolytic oxygen generator based on the use of palladium and by a new method of measuring the oxygen added. The apparatus was designed to measure the oxygen uptake by 5–10 g. of peas and has been extensively used in conjunction with an automatic method for measuring the carbon dioxide produced (Wager, 1957). Recently it has been used to determine the oxygen uptake by 100–150 g. of potatoes from atmospheres of different oxygen content (Mapson & Burton, 1962).

METHODS

Principle of the method (*Fig.* 1)

The tissue is enclosed in an airtight respiration chamber above a solution of sodium hydroxide, which serves to absorb the carbon dioxide given out. This chamber is connected to a compensating volume through a narrow U-tube manometer and also to an electrolytic oxygen generator (Figs. 1 and 3). A platinum electrode is fixed

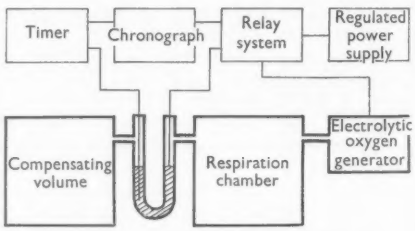

Fig. 1. Block diagram of the apparatus.

just above the meniscus in one limb of the manometer and as the tissue absorbs oxygen the meniscus rises and finally touches the electrode. At regular intervals the manometer is tested for electrical continuity by the 'timer' and when it occurs the relay system switches a constant current through the oxygen generator for a standard time. The 'pulse' of oxygen so produced blows the meniscus clear of the electrode. Since each pulse of oxygen is of equal volume the rate of respiration is calculated from their frequency as recorded by the chronograph.

During a run there is no significant change in partial pressure of oxygen in the respiration chamber (pulse volume : chamber volume is 1:6000), and the apparatus therefore can measure oxygen uptake from gas mixtures containing any partial pressure of oxygen up to 1 atm.

Description of the apparatus

Oxygen generator. The basic requirements of the oxygen generator are that it must produce pure oxygen in exact equivalence to the current passed, and it must be sealed from the atmosphere. The electrolysis of alkaline copper sulphate was found to produce some ozone and to be liable to side reactions. A satisfactory alternative is to electrolyse a solution of sodium hydroxide by using a large palladium cathode which absorbs the hydrogen produced. All parts of the cathode must receive an equal current, the amount of palladium must be sufficient to absorb all the hydrogen as it is produced (this is considerably less than the total hydrogen capacity) and the movement of oxygenated electrolyte to the cathode must be restricted. These requirements are met by the generator shown in longitudinal section in Fig. 2. It consists of two 35 mm. flat glass joints separated by and clamped to a polystyrene plate through which passes a 2–3 mm. bore glass tube sealed with a sintered-glass disk. The cathode consists of 25 g. of 1 mm. diam. palladium wire wound into a concave spiral and bedded down on a polythene mount. The concavity is such that all parts of the palladium spiral are approximately equidistant from the exit to the anode compartment and hence receive equal current. (The spiral is held together by 'spokes' of palladium wire on the outer side.) This cathode absorbs reliably about 200 ml. of hydrogen and the generator therefore can deliver up to 100 ml. of oxygen to the respiration chamber. The hydrogen is discharged from the palladium by disconnecting the generator from the respiration chamber and passing an electric current through it in the reverse direction until the original cathode just evolves oxygen. The anode consists of 2–3 cm. of 1 mm. diam. palladium wire, which in use rapidly becomes black.

These generators do not appear to produce ozone mixed with the oxygen, probably because the oxygen is formed on a blacked palladium electrode.

Two similar generators are used in series, one connected to the respiration chamber and one to a gas burette so that a running check can be kept on the oxygen generated and hence of the size of each pulse of oxygen.

Respiration chamber and manometer. The respiration chamber is rigidly constructed of glass and polythene of a shape suitable to the tissue to be used and with a lid, which, after sealing, can be clamped to the base. This chamber, the manometer bridge, the compensating volume and the oxygen generator are made with all-glass connexions and mounted rigidly on a small stand (Fig. 3).

A volume change of less than 0·1 ml. in 600 ml. causes the electrode in the manometer to make or break contact with

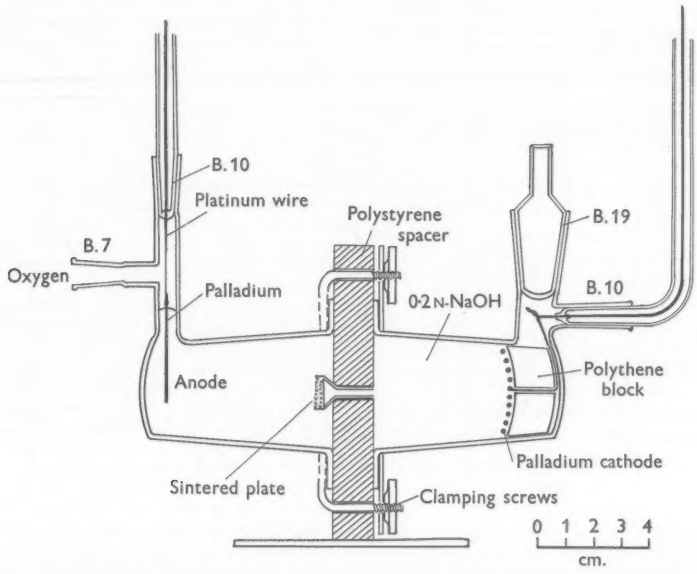

Fig. 2. Diagrammatic longitudinal section of the electrolytic oxygen generator.

the meniscus. To obtain this sensitivity one limb of the manometer is constricted to an internal diameter of 4 mm. and the electrode consists of 5 mm. of 0·004 mm. diam. platinum wire welded to a basal portion of about 1 mm. diam. platinum wire.

For satisfactory operation the manometer fluid must wet the glass, have a low surface tension and density and a reasonably good electrical conductance; a solution of 1% sodium carbonate in 50% (v/v) ethanol–water is suitable.

Electrical circuits (*Fig.* 4). The electrical circuits have to switch on the electrolytic oxygen generator for a standard time when the electrode in the manometer makes contact with the meniscus, to record the time at which this occurs and to operate with the small current that it is practicable to pass through the manometer fluid.

These requirements are met by using a monostable valve relay circuit which can be triggered into its unstable state when the manometer circuit is complete. A test of the

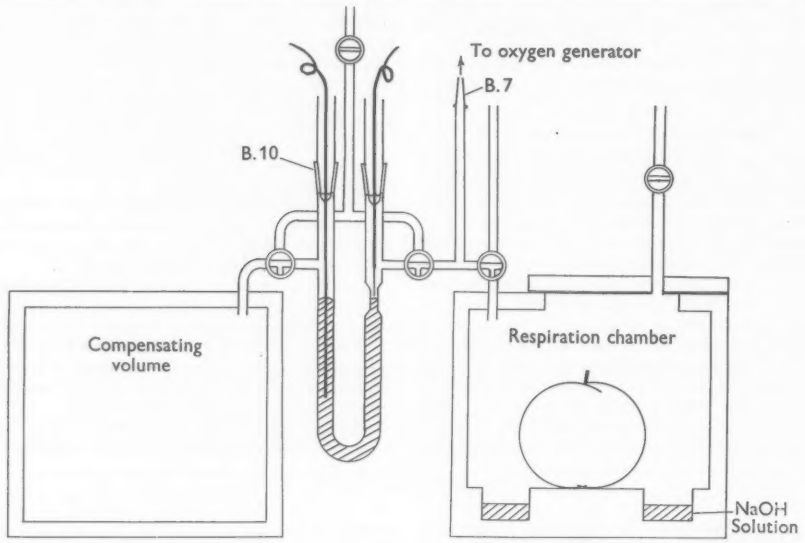

Fig. 3. Diagram of the manometer with its connexions to the respiration chamber and compensating volume.

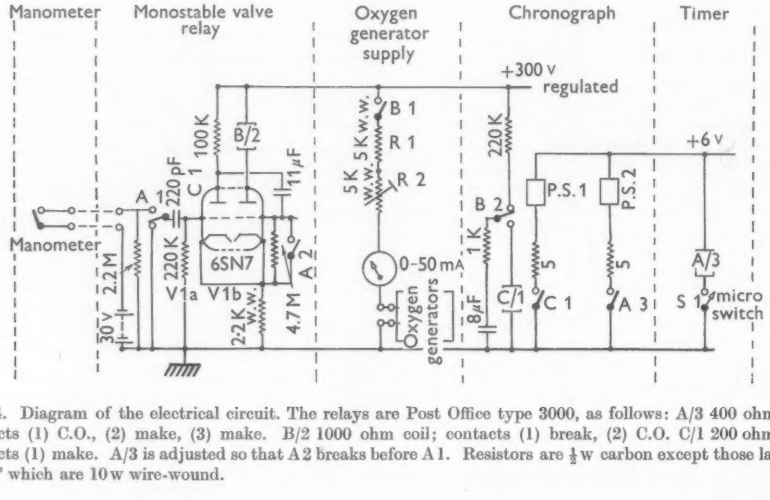

Fig. 4. Diagram of the electrical circuit. The relays are Post Office type 3000, as follows: A/3 400 ohm coil; contacts (1) C.O., (2) make, (3) make. B/2 1000 ohm coil; contacts (1) break, (2) C.O. C/1 200 ohm coil; contacts (1) make. A/3 is adjusted so that A 2 breaks before A 1. Resistors are ½ w carbon except those labelled 'w.w.' which are 10 w wire-wound.

manometer circuit is made at intervals of 1 min. by the 'timer', which consists of a micro-switch S1 operated by a cam driven at 1 rev./min. by the synchronous motor that also drives the chronograph.

In the stable condition the grid of valve V1a is more negative than the grid of V1b, and therefore current flows preferentially through V1b, the relay B/2 is energized and the contacts B1 are open. When the micro-switch S1 makes, the relay A/3 is energized, contacts A1 change over to connect the manometer while contacts A2 ensure that the circuit is in its stable condition. If at this time the electrode in the manometer is making contact with the meniscus, current flows through the manometer fluid and the capacitor C1 becomes charged. When the micro-switch S1 opens, the contacts A2 open first, then the contacts A1 connect the capacitor C1 to ground, thus placing a positive-going pulse on to the grid of V1a which causes this valve to pass current instead of V1b. The relay B/2 therefore de-energizes and the contacts B1 apply a voltage to the oxygen generators. The contacts B2 in conjunction with the relay C/1 energize pen solenoid P.S.1 for a brief time to mark the chronograph chart.

After 1 min. has passed the micro-switch again makes and the relay contacts A2 close, restoring the circuit to its stable state and switching off the oxygen generators.

The current through the generators is adjusted by R1+R2 to a value of 27·2 mA (equivalent to 0·1 ml. of O_2/min. at 15° and 760 mm.).

A power supply unit (not shown) supplies the current for the relays as well as a 300v regulated supply for the valves and the oxygen generators.

The chronograph was made for a 1·5v supply [C. F. Palmer (London) Ltd.; six-pen model] and is used with the 6v supply by adding 5Ω resistors in series with the solenoids.

Procedure. The T taps on the manometer bridge (Fig. 3) are turned so as to interconnect the compensating volume, the manometer bridge and the respiration chamber. Sufficient sodium hydroxide solution (0·1–1·0N) to absorb all the carbon dioxide produced during the course of the run is put into the base of the respiration chamber, the plant material inserted and the lid sealed on and clamped down. The oxygen generator is connected to the respiration chamber and the whole unit (respiration chamber, manometer, compensator and generator) immersed in a constant-temperature water bath (±0·01°) and left to attain temperature equilibrium (1–2 hr.). At the end of this time the compensating volume and manometer are shut off from the respiration chamber and the latter is flushed with gas of the required partial pressure of oxygen, equilibrated to the temperature of the water bath and to a relative humidity of about that above the solution of sodium hydroxide in the respiration chamber. The respiration chamber is then finally closed to the atmosphere and by manipulation of the taps on the manometer unit the compensating volume is first brought to the same pressure as the respiration chamber and then connected to it via the manometer only.

The electrode in the manometer is set to just make contact with the meniscus when there is no pressure difference across the manometer, with the result that at least one pulse occurs when the oxygen generator and relay are switched on and thereafter the pulse rate settles down to correspond to the true respiration rate.

DISCUSSION

The respiration chambers so far used have had volumes of about 600 ml. and the addition of an oxygen pulse of 0·1 ml. has blown the meniscus in the manometer clear of the electrode. This pulse volume is relatively large for a slowly respiring tissue and could be reduced to some extent but the limiting conditions have not been studied.

Temperature fluctuations of the water bath must be very small. The temperature variations as the heater comes on and off are smoothed out by constructing the chamber of relatively thick insulating material. Without this the time of individual pulses would vary but an average of a number would give the true rate of respiration.

The manometer is only tested for electrical continuity at intervals of 1 min. and a pulse of oxygen is produced at the first whole minute after contact between the meniscus and the electrode has been made. This limits the accuracy over short periods. If this error were important the frequency of testing could be increased with a corresponding increase in current to the generator, but alteration in this direction is limited by the rate of absorption of hydrogen by the cathode.

For certain tissues, e.g. apples, the use of a closed chamber is unsatisfactory because of the accumulation of 'volatiles', but the effect can be virtually eliminated by periodically flushing the respiration chamber with the appropriate gas mixture.

At very low partial pressures of oxygen, particularly with actively respiring material, a magnetic stirrer should be incorporated in the respiration chamber to reduce the diffusive resistance of the system.

The accuracy of the apparatus has been tested by setting it up without plant material and connecting the tap on the roof of the chamber to a small gas pipette immersed in the water bath. The gas pipette was initially filled with mercury which was allowed to flow out slowly (1–2 ml./hr.). After 15–18 ml. had been removed the flow was stopped and the apparatus allowed to come to equilibrium. The volume removed was calculated from the weight of mercury removed and this was compared with the volume of oxygen produced by the external generator, allowance being made for water vapour pressure, temperature and barometric pressure changes. The recovery from the external generator in several experiments was within 0·1 ml. of the calculated volume of mercury.

SUMMARY

1. An apparatus for recording the oxygen uptake by whole plant organs is described. The respired carbon dioxide is absorbed by a solution of sodium

hydroxide and the oxygen taken up is replaced by electrolytically generated oxygen, which is added in small equal-sized volumes. The frequency of their addition is a measure of the respiration rate.

2. The oxygen is produced by electrolysis of a solution of sodium hydroxide by using a large palladium cathode to absorb the hydrogen.

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Studies in the Biochemistry of Micro-organisms

109. THE STRUCTURE OF ASPERTHECIN*

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Asperthecin, $C_{15}H_{10}O_8$, the polyhydroxyanthraquinone derived from *Aspergillus quadrilineatus* Thom & Raper, was first isolated by Howard & Raistrick (1955). They prepared various derivatives and advanced a partial structure based on emodin. As a result of further degradative work by Neelakantan, Pocker & Raistrick (1957), the structure of asperthecin was reduced to two possibilities, (I) and (II), but the evidence was insufficient to determine which was correct.

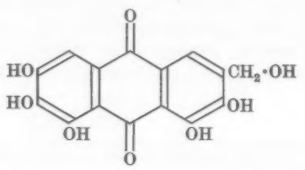
Further work on asperthecin, here presented, has afforded proof that the structure of asperthecin is represented by (II).

The evidence previously available, supporting structure (II), was mainly based on the visible absorption spectrum. The location of the main absorption band in this region at $\lambda_{484\,m\mu}$ (Birkinshaw, 1955) is strong evidence for the presence of three α-hydroxyl groups (cf. Birkinshaw & Gourlay, 1961). A further indication in favour of structure (II) was the fact that methylation of asperthecin with diazomethane in ether for a limited period afforded a di-*O*-methylasperthecin. Prolonged methylation in presence of methanol produced tetra- and penta-methylasperthecin, but a trimethylasperthecin was not isolated (Neelakantan *et al.* 1957). This suggests that asperthecin has only two β-hydroxyl groups.

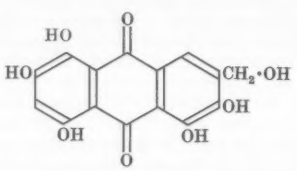
The main evidence against structure (II) and favouring structure (I) was the fact that solutions of asperthecin showed no fluorescence in daylight. Since fluorescence was considered to be diagnostic for quinizarin derivatives the presence of a 1,4 arrangement of hydroxyl groups as in structure (II) was unlikely (Howard & Raistrick, 1955).

Further evidence favouring structure (II) has now been obtained by partial (short-time) acetylation with acetic anhydride, by the method used in investigating dermocybin (Birkinshaw & Gourlay, 1961). After 12 min. in boiling acetic anhydride asperthecin afforded mainly a red triacetate with some orange tetra-acetate. The first stage of acetylation should be confined to the nuclear β-hydroxyl groups and the ω-hydroxyl group. Since a triacetate is first formed, the inference is that asperthecin has only two β-hydroxyl groups and hence three α-hydroxyl groups. In accordance with this, triacetylation of asperthecin produces only a small shift in the main visible absorption maximum from 484 to 478 mμ, the latter value being still

* Part 108: Stickings & Townsend (1961).



(I)



(II)

within the range expected for three free α-hydroxyl groups. The absorption peaks in both the visible and ultraviolet spectra of triacetylasperthecin, which, on the basis of structure (II), should have free hydroxyl groups in the 4,5,8 positions, are very close to those obtained for helminthosporin (4,5,8-trihydroxy-2-methylanthraquinone) and islandicin (1,4,5-trihydroxy-2-methylanthraquinone) (Birkinshaw, 1955).

The production of a tetra-acetate as the next stage in acetylation would then be due to acetylation of an α-hydroxyl group. Since, when three α-hydroxyl groups are present, two must occupy positions *peri* to one and the same carbonyl group, the hydrogen-bonding of each member of this pair is correspondingly weakened, rendering one of these two hydroxyl groups more susceptible to acetylation. In accordance with this the tetra-acetate shows visible absorption at $\lambda_{max.}$ 432 mμ, indicative of two free α-hydroxyl groups [cf. anthrarufin (1,5-dihydroxyanthraquinone), $\lambda_{max.}$ 430 mμ; emodin, $\lambda_{max.}$ 437 mμ]. This behaviour is to be expected from structure (II), whereas structure (I), with three β-hydroxyl groups and one ω-hydroxyl group, should most readily afford a tetra- and then a penta-acetate.

It is not possible to say which of the hydroxyl groups 4 and 5 is acetylated in the tetra-acetate. The fact that this acetate in acetic acid shows daylight fluorescence would suggest that it has free hydroxyl groups in the 5,8 (quinizarin) positions, which would indicate acetylation of the 4-hydroxyl group. A comparison of the visible and ultraviolet-absorption peaks, however, indicates that the tetra-acetate is more closely related to anthrarufin (1,5-dihydroxyanthraquinone) than to quinizarin (see Table 1). This evidence favours acetylation of the 5-hydroxyl group leaving free the 4- and 8-hydroxyl groups.

The weight of evidence in favour of structure (II) led us to attempt to confirm this structure by synthesis. In order to simplify this approach the hydroxymethyl group was eliminated from asperthecin by oxidation of the group to a carboxyl group with nitrous acid in sulphuric acid by the method of Hill & Richter (1936), to yield asperthecic acid, $C_{15}H_8O_9$, followed by decarboxylation of the asperthecic acid to the pentahydroxyanthraquinone. Synthesis of the latter substance was achieved as follows.

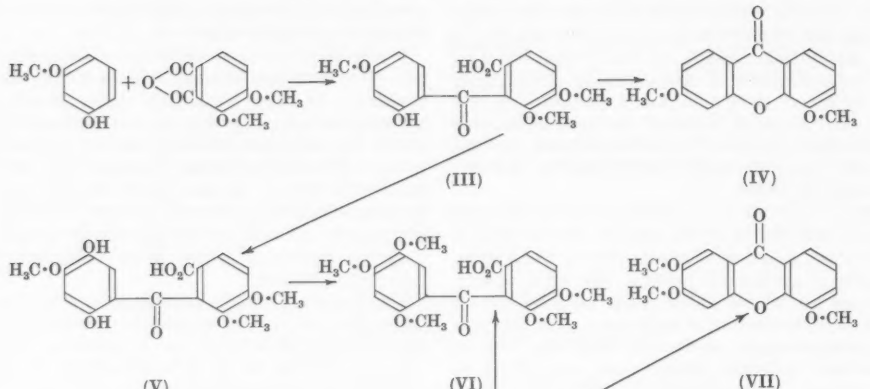
The first stage was the condensation of hemipinic anhydride with *m*-methoxyphenol (present in large excess to act as solvent) in the presence of aluminium chloride to form 2-(2′-hydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (III). The method of linkage of the two moieties was settled by decarboxylation in quinoline with a copper chromite catalyst, which also caused monodemethylation and ring-closure with the production of a dimethoxyxanthone that must have structure (IV). 3,5-Dimethoxyxanthone (IV) has been synthesized by Noyce & Weldon (1952). The production of this xanthone decides the manner of linkage of the two benzene nuclei in the benzoylbenzoic acid (III), since the new ether linkage must have occurred (with elimination of methanol) between a hydroxyl and a methoxyl group, both *ortho* to the common carbonyl link.

Oxidation with alkaline persulphate (Elbs method) converted (III) into the 2-(2′,5′-dihydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (V), affording the pentamethoxy derivative (VI) on methylation. It was then found that a more direct approach was possible, since by allowing hemipinic

Table 1. *Light-absorption spectra of polyhydroxyanthraquinones in ethanol*

	Substituted anthraquinones	$\lambda_{max.}$ (mμ) with log ε (in parentheses)						
1	Triacetylasperthecin	233 (4·37)	256 (4·28)	290* (4·00)	—	—	478 (3·93)	545–555* (3·24)
2	Tetra-acetylasperthecin	229 (4·61)	255 (4·38)	280–290* (4·20)	—	432 (4·06)	—	—
3	Anthrarufin (1,5-dihydroxyanthraquinone)	225 (4·64)	253 (4·31)	286 (4·02)	—	430 (4·04)	—	—
4	Quinizarin (1,4-dihydroxyanthraquinone)	225 (4·34)	249 (4·49)	279 (4·01)	325 (3·39)	450 (3·93)	—	—
5	Asperthecic acid	221 (4·20)	245 (4·33) 269 (4·38)	298 (4·09)	388 (3·49)	—	491 (4·18)	515 (4·11)
6	Decarboxyasperthecic acid	238 (4·44)	259 (4·46)	286·5 (4·14)	311 (4·03)	—	485 (4·25)	512 (4·22)
7	3,4,5,7,8-Pentahydroxyanthraquinone	238 (4·44)	259 (4·45)	286·5 (4·15)	311 (4·02)	—	485 (4·25)	512 (4·21)

* Inflexion.



(III) (IV) (V) (VI) (VII) (VIII) (IX)

anhydride to react with 1,2,4-trimethoxybenzene monodemethylation occurred and a monohydroxytetramethoxybenzoylbenzoic acid (VIII) was obtained. A by-product of this reaction, formed in considerable yield, appeared from its analysis to be 3,4-dimethoxy-1,2-di-(2′,4′,5′-trimethoxybenzoyl)-benzene. The hydroxytetramethoxybenzoylbenzoic acid (VIII) gave on methylation the same pentamethoxybenzoylbenzoic acid (VI) as had been obtained by the method involving the Elbs oxidation. The conversion of the acid (VIII) into a trimethoxyxanthone (VII) by decarboxylation (with elimination of methanol) indicated that demethylation involved in the synthesis of the benzoylbenzoic acid had occurred at one of the groups *ortho* to the carbonyl link. Since the hemipinic anhydride moiety had not been demethylated when condensed with *m*-methoxyphenol, it appears to be stable to the reaction conditions and the demethylation must have occurred on the trimethoxybenzene moiety. Hence the hydroxytetramethoxybenzoylbenzoic acid is formulated as (VIII).

Treatment of (VIII) with a fused mixture of aluminium chloride and sodium chloride afforded the 3,4,5,7,8-pentahydroxyanthraquinone (IX), which was purified through the penta-acetate. On comparison of properties and absorption spectrum of this synthetic product with those of decarboxyasperthecic acid, the two were found to be identical. The melting points of their penta-acetates agreed and were not depressed on mixing. Asperthecin, therefore, has the structure 3,4,5,7,8-pentahydroxy-2-hydroxymethylanthraquinone (II).

The synthetic 3,4,5,7,8-pentahydroxyanthraquinone and other pentahydroxyanthraquinones synthesized (Birkinshaw & Gourlay, 1961), namely 1,4,5,6,7-pentahydroxy-2-methyl-, 1,4,6,7,8-pentahydroxy-2-methyl-, 4,5,6,7,8-pentahydroxy- and 4,5,6,7,8-pentahydroxy-2-methyl-anthraquinone, all have the quinizarin grouping, yet none of them showed any daylight fluorescence when dissolved in acetic acid, and in this respect behaved like asperthecin and asperthecic acid. The absence of fluorescence, therefore, can no longer be regarded as a reliable indication of the absence of a quinizarin grouping in the polyhydroxyanthraquinone molecule.

EXPERIMENTAL

The C and H determinations were made by Dr A. Schoeller; OMe and acetyl by one of us (R.G.). All melting points are uncorrected. The light-absorption values of the various derivatives and synthetic products, where not given below, are recorded in Table 1.

Derivatives of asperthecin

Triacetylasperthecin. Asperthecin (0·1 g.) was refluxed with acetic anhydride (5 ml.) for 8 min., when solution was effected. After refluxing for a further 4 min. the solution was cooled and a mixture of red and orange crystals (0·11 g.) separated. Recrystallization from acetic anhydride, avoiding excessive heating, afforded *triacetylasperthecin* as bright-red needles (0·08 g.), m.p. 248–250°

(decomp.) (Found: C, 55·3; H, 3·7; acetyl, 29·0. $C_{21}H_{16}O_{11}$, $\frac{1}{2}H_2O$ requires C, 55·6; H, 3·8; 3 acetyl, 28·5%).

Tetra-acetylasperthecin. The mother liquors from the preceding reaction, when poured into water, gave an orange–red precipitate, which was repeatedly crystallized from acetic acid, giving *tetra-acetylasperthecin* as bright-orange needles (0·02 g.), m.p. 251–253° (decomp.). The tetra-acetate shows slight but distinct fluorescence when dissolved in acetic acid. An improved yield (0·07 g.) of the tetra-acetate was obtained by refluxing asperthecin (0·1 g.) with acetic anhydride (5 ml.) for 20 min. (Found: C, 56·5; H, 3·7; acetyl, 35·2. $C_{23}H_{18}O_{12}$ requires C, 56·8; H, 3·7; acetyl, 35·4%).

Oxidation of asperthecin; asperthecic acid penta-acetate. Asperthecin (0·2 g.), H_3BO_3 (1 g.), $NaNO_2$ (0·5 g.) and conc. H_2SO_4 (25 ml.) were heated rapidly to 180° (oil-bath temp.), cooled to 145° and maintained at 145° for 80 min. The cooled solution was poured into water (250 ml.) and heated to boiling to coagulate the product, and the ppt. (0·17 g.) filtered off and dried. The crude product was acetylated by heating with acetic anhydride (5 ml.) and conc. H_2SO_4 (0·1 ml.) at 90° for 5 min. The solution was cooled and poured into water and the brown ppt. (0·19 g.), m.p. 190–193°, was filtered off, dried and recrystallized three times from methanol, giving *asperthecic acid penta-acetate* (0·05 g.), m.p. 218–220° (decomp.) (Found, on sample dried at 100° *in vacuo*: C, 55·0; H, 3·6; acetyl, 40·4. $C_{25}H_{18}O_{14}$ requires C, 55·3; H, 3·3; 5 acetyl, 39·7%.)

Asperthecic acid. The penta-acetate (0·06 g.) was hydrolysed by refluxing with methanol (5 ml.) containing conc. H_2SO_4 (0·15 ml.). The acetate dissolved and after heating for 1 hr. red crystals separated. The product was filtered off and washed with methanol giving *asperthecic acid* (30 mg.), m.p. (decomp.) above 350° (Found, on material dried at 100° *in vacuo*: C, 54·5; H, 2·7. $C_{15}H_8O_9$ requires C, 54·2; H, 2·4%).

Decarboxylation of asperthecic acid. Asperthecic acid (0·35 g.), quinoline (15 ml.) and copper chromite (0·2 g.) were heated to 200° in a current of N_2. The effluent gases were passed through standard aq. $Ba(OH)_2$ solution. The reaction as measured by CO_2 evolved was 65% complete in 7½ hr. and was stopped at this stage. The cooled solution was added to 2N-HCl (250 ml.), and the ppt. filtered off and dried. The product was extracted with methanol (3 × 150 ml.). The orange methanolic solution was evaporated to 200 ml. and chromatographed on a column of $CaCO_3$. Ethanol was used as the eluent. Two distinct coloured bands were obtained, an orange and a deep pink, shading to violet towards the base. The column was removed and cut into three portions, orange, pink and violet, and each portion dissolved in 5N-HCl. The solutions were heated to boiling and cooled, and the anthraquinone fractions filtered off and dried giving fraction 1, 0·07 g., fraction 2, 0·10 g. and fraction 3, 0·04 g. Purification of fractions 2 and 3 by acetylation gave the acetyl derivatives, m.p. 242–243°, undepressed when mixed with each other or with an authentic sample of 3,4,5,7,8-penta-acetoxyanthraquinone. The acetate (60 mg.) was hydrolysed by refluxing for 1½ hr. with methanol (5 ml.) and conc. H_2SO_4 (0·1 ml.). *Decarboxyasperthecic acid* was precipitated as red needles, m.p. 353–354°. No depression in melting point occurred when this material was mixed with synthetic 3,4,5,7,8-pentahydroxyanthraquinone (Found, on sample dried *in vacuo* at 100°: C, 58·35; H, 2·8. $C_{14}H_8O_7$ requires C, 58·3; H, 2·8%). Compare u.v. spectra in Table 1.

Syntheses

2-(2′-Hydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (III). Hemipinic anhydride (from hemipinic acid, 5 g.) was dissolved in *m*-methoxyphenol (23 g.). Powdered anhydrous $AlCl_3$ (8 g.) was added in portions and after the initial reaction had subsided the mixture was heated at 70–75° (oil-bath temp.) for 6 hr. After standing overnight, the viscous red mass was treated with ice and 2N-HCl (50 ml.) and the mixture extracted with ether (3 × 150 ml.). The ether was washed twice with $NaHCO_3$ solution (100 ml., 50 ml. 10%). Acidification of the bicarbonate extract precipitated a resinous material. This material when dissolved in ether (200 ml.) and the volume reduced to 50 ml. gave a pale-yellow crystalline compound (2·2 g.; m.p. 204–207°). One recrystallization from methanol gave needles of 2-(2′-*hydroxy-4′-methoxybenzoyl*)-3,4-*dimethoxybenzoic acid*, m.p. 211–212°, raised by sublimation to 215° (Found, on sublimed sample: C, 61·0; H, 4·8; OMe, 27·8. $C_{17}H_{16}O_7$ requires C, 61·4; H, 4·8; OMe, 28·0%).

2-(2′,5′-Dihydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (V). 2-(2′-Hydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (III) (2·20 g.) was dissolved in water (20 ml.) containing NaOH (1·33 g.). $K_2S_2O_8$ (1·80 g.) was added in portions to the solution at 35–40° during 2·5 hr. After 4 days at room temp. the deep-red solution was made faintly acid to Congo red with 5N-H_2SO_4. The unchanged acid (1·35 g.), m.p. 203–206°, was collected and the filtrate was made strongly acid with conc. HCl (10·0 ml.). The crystalline product (0·66 g.), m.p. 225–231°, was collected after 3 days. Recrystallization from methanol–water afforded 2-(2′,5′-*dihydroxy-4′-methoxybenzoyl*)-3,4-*dimethoxybenzoic acid* (V) as prisms, m.p. 257–258° (decomp.), raised by sublimation to 261–262° (decomp.) (Found: C, 58·5; H, 4·7; OMe, 26·7. $C_{17}H_{16}O_8$ requires C, 58·6; H, 4·6; OMe, 26·7%).

3,4-Dimethoxy-2-(2′,4′,5′-trimethoxybenzoyl)benzoic acid (VI). 2-(2′,5′-dihydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (0·15 g.) was dissolved in acetone (20 ml.) and K_2CO_3 (1 g.) and dimethyl sulphate (1·0 ml.) were added. The solution was refluxed for 7 hr., kept overnight and then refluxed for a further 7 hr. after the addition of dimethyl sulphate (1·0 ml.). The cooled solution was filtered and the acetone evaporated to give a pale-yellow oil. The oil was suspended in 2N-NaOH (25 ml.) and aq. 2N-NH_3 (5 ml.) was added. The solution was refluxed for 2 hr. to hydrolyse any ester, cooled, acidified and extracted with ether. A colourless solid (0·074 g.), m.p. 227–229°, was isolated from the ether extract. Two recrystallizations from methanol gave colourless needles (0·032 g.), m.p. 230–231°, of 3,4-*dimethoxy*-2-(2′,4′,5′-*trimethoxybenzoyl*)-*benzoic acid* (Found, on sample dried at 100° *in vacuo*: OMe, 41·6. $C_{19}H_{20}O_8$ requires OMe, 41·2%).

Decarboxylation of 2-(2′-hydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid; 3,5-dimethoxyxanthone (IV). The benzoylbenzoic acid (1·1 g.), quinoline (12 ml.) and copper chromite (0·4 g.) were heated at 190–200° under the same conditions as above. The reaction was 80% complete in 4 hr., when it was stopped. The cooled solution was poured into 2N-HCl (100 ml.) and the mixture extracted with ether (3 × 100 ml.). The ether solution was reduced in volume and the pale-yellow solid which separated crystallized from ethanol in long colourless needles (0·15 g.), m.p. 170–171°, of 3,5-dimethoxyxanthone. Noyce & Weldon (1952)

report the m.p. of synthetic 3,5-dimethoxyxanthone as 173·5–174·5° (corr.). (Found: C, 70·4; H, 4·7; OMe, 24·4. $C_{15}H_{12}O_4$ requires C, 70·3; H, 4·7; OMe, 24·2%). Light-absorption values in ethanol: $\lambda_{max.}$ 236, 246, 267, 304 and 330 mμ; log ε 4·51, 4·52, 4·24, 4·10 and 3·85 respectively.

2-(2′-*Hydroxy*-4′,5′-*dimethoxybenzoyl*)-3,4-*dimethoxybenzoic acid* (VIII). Hemipinic anhydride (from hemipinic acid, 2 g.) was dissolved in 1,2,4-trimethoxybenzene (10 ml.) by warming. The solution was cooled rapidly to room temp., powdered anhydrous $AlCl_3$ (4·0 g.) was added and the mixture warmed at 75–80° (oil-bath temp.) for 5½ hr. After 18 hr. at room temp. the viscous green product was warmed with 2N-HCl (50 ml.), cooled and extracted with ether. The ether solution was shaken with $NaHCO_3$ solution (2 × 100 ml., 10%). A crystalline solid separated from the ether during the extraction. This product crystallized from ethanol in colourless needles, m.p. 176° (Found, on sample dried *in vacuo* at 60°: C, 63·8; H, 5·6; OMe, 46·9; mol.wt. 540. $C_{28}H_{30}O_{10}$ requires C, 63·8; H, 5·7; 8 OMe, 47·1%; mol.wt. 526). This by-product appears to be 3,4-dimethoxy-1,2-di-(2′,4′,5′-trimethoxybenzoyl)benzene. The resinous product obtained on acidification of the $NaHCO_3$ solution was dissolved in ether. The ether was evaporated to small volume, when a pale-yellow material separated. Crystallization from methanol–water afforded a cream-coloured product (0·75 g.), m.p. 225°, consisting of 2-(2′-*hydroxy*-4′,5′-*dimethoxybenzoyl*)-3,4-*dimethoxybenzoic acid* (Found, on sample dried at 100° *in vacuo*: C, 59·6; H, 4·9; OMe, 34·3. $C_{18}H_{18}O_8$ requires C, 59·7; H, 5·0; OMe, 34·3%).

Methylation of the hydroxytetramethoxybenzoylbenzoic acid. The pentamethyl ether (VI) of the hydroxytetramethoxybenzoylbenzoic acid was prepared by the method used for the methylation of 2-(2′,5′-dihydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid. The product was isolated and crystallized from methanol, giving colourless needles, m.p. 230–231°, undepressed when mixed with the methylation product of 2-(2′,5′-dihydroxy-4′-methoxybenzoyl)-3,4-dimethoxybenzoic acid (Found, on sample dried at 100° *in vacuo*: C, 60·5; H, 5·4; OMe, 41·4. $C_{19}H_{20}O_8$ requires C, 60·6; H, 5·4; OMe, 41·2%).

Decarboxylation of 2-(2′-*hydroxy*-4′,5′-*dimethoxybenzoyl*-3,4-*dimethoxybenzoic acid; 2,3,5-trimethoxyxanthone* (VII). The benzoylbenzoic acid (1·25 g.), quinoline (12·5 ml.) and copper chromite (0·4 g.) were heated at 200° in an atmosphere of N_2 for 4½ hr. The effluent gases were passed through standard aq. $Ba(OH)_2$ solution. The CO_2 produced in this time was 72% of the theoretical amount. The cooled solution was poured into 2N-HCl (250 ml.) and the mixture was filtered. The dried residue was extracted with ether, and the ether extract washed with bicarbonate and evaporated to small volume, when a cream-coloured solid separated. Recrystallization from methanol gave 2,3,5-*trimethoxyxanthone* as cream-coloured needles, m.p. 232–235°. Sublimation raised the m.p. to 238° (Found, on sublimed sample dried at 100° immediately before analysis: C, 66·6; H, 4·8; OMe, 32·1. $C_{16}H_{14}O_5$ requires C, 67·1; H, 4·9; OMe, 32·5%). Light-absorption values in ethanol: $\lambda_{max.}$ 219, 249, 314 and 330 mμ; log ε 4·39, 4·68, 4·00 and 4·05 respectively.

3,4,5,7,8-*Penta-acetoxyanthraquinone.* $AlCl_3$ (5 g.) and NaCl (1 g.) were fused at 180°, cooled to 120° and 2-(2′-hydroxy-4′,5′-dimethoxybenzoyl)-3,4-dimethoxybenzoic acid (0·56 g.) was added with stirring. The flask was transferred to an oil bath at 190° and heating continued for 5 min. The cooled mixture was treated with ice and conc. HCl (20 ml.) and diluted with water (400 ml.). The ppt. coagulated on boiling and was filtered off, washed and dried. The brown product (0·38 g.) was heated for 10 min. at 70–80° with acetic anhydride (5 ml.) containing conc. H_2SO_4 (0·1 ml.). On cooling, pale-yellow needles separated. The mixture was filtered and the product (0·39 g.) recrystallized from methanol, affording lemon-yellow needles (0·32 g.) of 3,4,5,7,8-*penta-acetoxyanthraquinone*, m.p. 241–242°. A further recrystallization raised the m.p. to 243° (Found, on sample dried to constant wt. *in vacuo* at 100°: C, 57·9; H, 3·9; acetyl, 42·1. $C_{24}H_{18}O_{12}$ requires C, 57·8; H, 3·6; 5 acetyl, 43·3%).

3,4,5,7,8-*Pentahydroxyanthraquinone.* The penta-acetoxyanthraquinone (0·27 g.) was refluxed for 2 hr. with methanol (25 ml.) containing conc. H_2SO_4 (0·75 ml.). The acetate dissolved and after 30 min. red needles separated. The ppt. was filtered off and washed with methanol, giving 3,4,5,7,8-pentahydroxyanthraquinone as light-red–brown needles (0·15 g.), m.p. 353–355° (Found, on sample dried at 100° *in vacuo*: C, 58·2; H, 2·9. $C_{14}H_8O_7$ requires C, 58·3; H, 2·8%).

SUMMARY

1. Several new derivatives of asperthecin have been prepared. Acetylation of asperthecin with acetic anhydride has given the triacetate, m.p. 248–250° (decomp.), and the tetra-acetate, m.p. 251–253° (decomp.). Asperthecic acid has been obtained by the oxidation (nitrous acid in concentrated sulphuric acid) of the hydroxymethyl group of asperthecin to a carboxyl group. Asperthecic acid has been decarboxylated to a pentahydroxyanthraquinone.

2. The condensation of hemipinic anhydride with 1,2,4-trimethoxybenzene has given 2-(2′-hydroxy-4′,5′-dimethoxybenzoyl)-3,4-dimethoxybenzoic acid. Subsequent ring closure and demethylation of the benzoylbenzoic acid has afforded 3,4,5,7,8-pentahydroxyanthraquinone, identical with decarboxyasperthecic acid.

3. Asperthecin is, therefore, represented by the structure 3,4,5,7,8-pentahydroxy-2-hydroxymethylanthraquinone.

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Synthesis of Cell Constituents from Glycine by a *Pseudomonas*

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Janke & Tayenthal (1937) identified glyoxylic acid as an intermediate in the degradation of glycine by *Pseudomonas fluorescens*. Campbell (1955) prepared cell-free extracts of a species of *Pseudomonas* which oxidized glycine to ammonia, carbon dioxide and water and showed that glyoxylic and formic acids were intermediates in the oxidation. Since glycine is converted into glyoxylate, it was suggested (Dagley, 1958; Callely & Dagley, 1959) that reaction sequences found in bacteria metabolizing glycollate (Kornberg & Gotto, 1959; Kornberg & Gotto, 1961*a*) and oxalate (Quayle & Keech, 1959; Quayle, Keech & Taylor, 1961) might also be used by bacteria when growing with glycine as sole carbon source. The present work supports the view that tartronic semialdehyde is a reaction intermediate in bacterial metabolism under these conditions.

MATERIALS AND METHODS

Maintenance and growth of organisms. The original culture of *Pseudomonas* (A) was a gift from Professor B. C. J. G. Knight and was one of four species isolated from poultry house deep-litter (Bachrach, 1957). Stock cultures were maintained on nutrient-agar slopes and when sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) could be kept for several months at 4° without desiccation.

For experimental use, cells were grown with forced aeration at 30° in a medium adjusted with NaOH to pH 7·2 and containing, per litre: KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 0·5 g.; glycine, 1·4 g. (0·02M); $MgSO_4,7H_2O$, 0·4 g. After growth for 18 hr. cells were harvested in a Sharples centrifuge and washed once by resuspension in phosphate buffer (KH_2PO_4; 2 g./l.; pH 7·2) and centrifuged at 3° for 10 min. at 4300*g*. Growth of cultures was followed by measurement of their turbidities in a Spekker photoelectric absorptiometer (Dagley, Dawes & Morrison, 1950).

Preparation of cell extracts. Freshly grown cells of *Pseudomonas* (A) were disintegrated either in the Hughes (1951) press or by exposure for 5 min. at 0° to the output of a MSE–Mullard ultrasonic disintegrator at an average frequency of 20 kcyc./sec. Each 10 g. of cells crushed in the press were ground with 1 g. of polishing alumina, grade 3/50 (Griffin and George Ltd., Manchester), and 25 ml. of buffer (KH_2PO_4; 2 g./l.; pH 7·2). This procedure facilitated both the dispersal of the crushed cells in the buffer and the subsequent clarification of the viscous extract by centrifuging at 3° and 10 500*g* for 45 min. Protein concentrations of extracts were determined by the method of Sols (1947).

Paper chromatography. Paper chromatography of carboxylic acids was carried out by the ascending method on Whatman no. 1 papers: one-dimensionally in phenol–formic acid–water (500:13:167, w/v/v; Kornberg, 1958), and two-dimensionally, first in this solvent and then in butanol–acetic acid–water (120:30:50, by vol.; Wood, 1958) or in ether–benzene–formic acid–water (70:30:14:10, by vol.) followed by propanol–formic acid–water (6:3:1, by vol.; Weimberg, 1959). For 2,4-dinitrophenylhydrazones of keto acids, ascending chromatograms were run on Whatman no. 4 papers: one-dimensionally in butanol–ethanol–$(NH_4)_2CO_3$ buffer (80:22:28, by vol.; Dagley, Fewster & Happold, 1952), and two-dimensionally, first in this solvent and then in butanol–benzene–$(NH_4)_2CO_3$ buffer (80:5:15, by vol.; Fewster & Hall, 1951). Radioactive compounds were located and identified on chromatograms by radioautography and co-chromatography with authentic unlabelled materials. Non-volatile carboxylic acids used as carriers were located by spraying with a 1% solution of aniline and xylose in 95% (v/v) methanol (Wood, 1958). Descending reverse-phase chromatograms of mono- and bis-2,4-dinitrophenylhydrazones were run on silicone-treated Whatman no. 1 paper in *N*-dimethylformamide–water–chlorobenzene–heptane (7:1:16:4, by vol.; Meigh, 1956).

Microassays of labelled compounds. Reactions with ^{14}C-labelled substrates were carried out in Pyrex glass tubes (8 mm. × 70 mm.) tightly plugged with non-absorbent cotton wool. Volumes of reactants, 0·02–0·10 ml., were accurately delivered into the tubes from Pasteur pipettes filled and discharged by manipulation of a screw clip around the rubber teat. The pipettes were calibrated from blood pipettes and checked by weighing the water delivered into capped polythene vessels (15 mm. × 30 mm.). The total volume of each complete reaction mixture was 0·2–0·3 ml. After incubation at 30°, reactions were stopped by placing the tubes in a boiling-water bath for 1 min. They were then cooled in ice, 0·02 ml. of 3N-HCl was added, and the precipitate removed by centrifuging. Usually, 0·05 ml. of liquid was applied to the chromatography paper. Radioactive spots were located by radioautography and eluted with water into capped polythene vessels, and volumes determined by weighing. Samples (0·02 ml.) were plated out on the smooth sides of 19 mm. disks of Whatman no. 1 paper, dried for 10 min. at a distance of 12 cm. from a 100w light bulb and counted in a Tracerlab S.C. 16G windowless flow counter.

Isotopic materials. All isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [1-^{14}C]glyoxylate was diluted with unlabelled glyoxylate for use and unless otherwise stated contained 8 μmoles/ml. with an activity of 1 μc/ml. Labelled glyceric acid was prepared in solution when required by the action

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of sonic extracts of *Pseudomonas* (A) on sodium glyoxylate and DPNH; the yield of [^{14}C]glycerate from sodium [1-^{14}C]glyoxylate was 95% of the theoretical value.

Other materials used. Sodium glyoxylate was prepared from tartaric acid by periodate oxidation (Radin, Metzler, Bloom & Westerfield, 1955) and purified by repeated crystallization from aqueous acetone. Reduced pyridine nucleotides were from Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP, ADP, coenzyme A, glycolaldehyde and glyceric acid were from L. Light and Co. Ltd., Colnbrook, Bucks. Barium 2-phosphoglycerate, barium 3-phosphoglycerate and barium silver phosphoenolpyruvate were from C. F. Boehringer und Soehne, Mannheim, Germany. We thank Professor Frank Dickens, F.R.S., for a gift of lithium hydroxypyruvate and Mr P. J. Chapman for disodium 2,4-dioxovalerate and methyl 2,4-dioxovalerate. All other reagents used were of the highest purity commercially available.

Determination of materials. Glycolaldehyde forms the same bis-2,4-dinitrophenylhydrazone as glyoxal, and the present method is a modification of the determination of diacetyl as its bis-2,4-dinitrophenylhydrazone (Neuberg & Strauss, 1945). To 3 ml. of a solution containing 0–0·2 μmole of glycolaldehyde was added 5 ml. of 0·2% 2,4-dinitrophenylhydrazine in 2N-HCl, and after incubation for 1 hr. at 100° the precipitated bis-2,4-dinitrophenylhydrazone was centrifuged with 0·2 g. of kaolin, washed once with 3 ml. of 2N-HCl, twice with water and drained. The precipitate was extracted twice with 5 ml. of 0·3% sodium ethylate solution at room temperature, the extracts were made up to 25 ml. with sodium ethylate solution, and the extinctions were read at 580 mμ in a spectrophotometer and referred to a curve prepared from standard glycolaldehyde solutions similarly treated.

Glyoxylate and pyruvate were determined by the method of Friedemann & Haugen (1943).

Determination of enzyme activities. Glyoxylate carboligase (Krakow & Barkulis, 1956; Krakow, Barkulis & Hayashi, 1961) was assayed by measurement either of the rate of disappearance of [1-^{14}C]glyoxylate or the rate of anaerobic evolution of CO_2 from glyoxylate. For the latter, double-armed Warburg flasks which contained in the main compartments 15 μmoles of phosphate buffer, pH 7·2, and enzyme solution were gassed with O_2-free N_2 and thermally equilibrated at 30°. The reaction was started by adding sodium glyoxylate from side arm 1 and stopped by tipping 0·2 ml. of 50% (w/v) trichloroacetic acid from side arm 2, when all CO_2 was expelled from solution. When DPNH was added it was placed in the side arm with glyoxylate. Volumes were made up to 3 ml. with distilled water.

Tartronic semialdehyde reductase (Kornberg & Gotto, 1961*b*) was assayed by measuring spectrophotometrically at 340 mμ the rate of oxidation of DPNH in the presence of an excess of the C_3 compound produced enzymically from sodium glyoxylate before addition of DPNH. There is strong evidence that this C_3 compound is tartronic semialdehyde.

RESULTS

Accumulation of pyruvate in cultures

Cells grown in glycine and mineral salts medium were harvested after 20 hr. in the stationary phase and resuspended in an equal volume of aerated growth medium at 30°. During a lag in growth of 4 hr. there accumulated in the culture a keto acid, the 2,4-dinitrophenylhydrazone of which appeared to be identical with that of pyruvate from its absorption spectrum in alkali and its behaviour on chromatograms in the solvents described by Dagley *et al.* (1952) and Fewster & Hall (1951). When growth began, pyruvate disappeared (Fig. 1). Cell suspensions taken at the end of the exponential phase of growth did not lag in a fresh medium and there was no accumulation of pyruvate.

Pyruvate might be formed from glycine by conversion into serine; however, serine-dehydrase activity (Binkley, 1943; Wood & Gunsalus, 1949) could not be detected when suspensions of washed cells grown with glycine were incubated anaerobically with 50 μmoles of L-serine/3 ml.

Oxidation of substrates by whole cells

Pyruvate, malate, fumarate, succinate, glycine, glyoxylate and formate were rapidly oxidized by

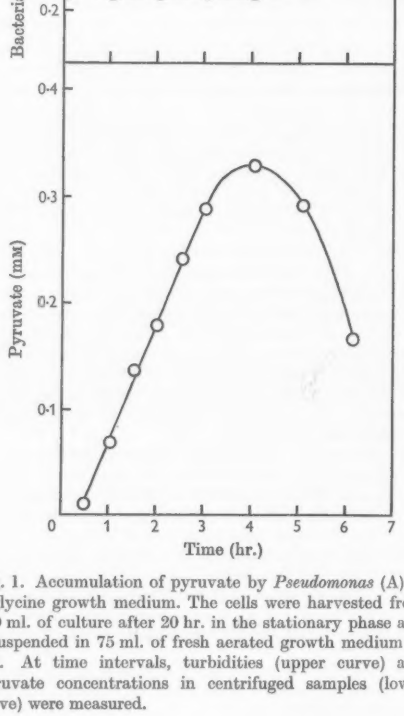

Fig. 1. Accumulation of pyruvate by *Pseudomonas* (A) in a glycine growth medium. The cells were harvested from 100 ml. of culture after 20 hr. in the stationary phase and resuspended in 75 ml. of fresh aerated growth medium at 30°. At time intervals, turbidities (upper curve) and pyruvate concentrations in centrifuged samples (lower curve) were measured.

cell suspensions taken at the onset of the stationary phase in glycine media. Acetate was oxidized rapidly after a lag of 10 min. in the respirometer and citrate slowly throughout; DL-alanine was oxidized rapidly and DL-serine slowly. Cells grown in succinate and mineral salts medium readily oxidized various compounds of the tricarboxylic acid cycle, except citrate, but glycine and glyoxylate were oxidized slowly.

Campbell (1955) showed that the oxidation of formate by extracts of another species of *Pseudomonas* was completely inhibited by addition of 2 mM-potassium hypophosphite, and glycine and glyoxylate were oxidized quantitatively to formate when hypophosphite was present. Addition of 2 mM-potassium hypophosphite inhibited oxidation of formate by whole cells of *Pseudomonas* (A) but not oxidation of glycine or glyoxylate, and no formate accumulated. Bachrach (1957) obtained similar results with whole cells of *Pseudomonas aeruginosa*. In contrast, oxidation of glycine and glyoxylate was decreased by the presence of inhibitors of the tricarboxylic acid cycle. Sodium fluoroacetate (0·1 mM) inhibited the oxidation of fumarate by washed cells completely and that of glycine and glyoxylate by 90%; in cultures, fluoroacetate (10 μM) inhibited growth on glycine by 93%. The oxidation of glycine by cell suspensions was reduced by 95 and 35% in the presence of 10 mM- and 1 mM-sodium arsenite respectively. In the second case there accumulated 0·39 μmole of pyruvate and 0·06 μmole of α-oxoglutarate/3 ml., the keto acids being identified by chromatography and spectrophotometry of their 2,4-dinitrophenylhydrazones. Cell extracts of *Pseudomonas* (A) oxidized neither formate nor glycine, but succinate and citrate were oxidized rapidly.

Enzymic decarboxylation of glyoxylate

Krakow & Barkulis (1956), Krakow *et al.* (1961) and Kornberg & Gotto (1959, 1961*a*) studied the decarboxylation of glyoxylate by extracts prepared respectively from *Escherichia coli* and from species of *Pseudomonas* grown with glycollate as carbon source; they found that 2 moles of glyoxylate gave rise to 1 mole of carbon dioxide by an enzyme glyoxylate carboligase. Callely & Dagley (1959) obtained yields of carbon dioxide greater than this but somewhat lower than required by the equation: 2 glyoxylate $\rightarrow$ glycolaldehyde + $2CO_2$. They suggested the existence of an enzyme in their cell extracts that slowly decarboxylated the C_3 intermediate as it accumulated from the more rapid action of the carboligase. This has been reinvestigated as follows. The evolution of carbon dioxide from glyoxylate, catalysed by a sonic extract of *Pseudomonas* (A) grown with glycine, was measured in a series of double-armed Warburg flasks, and the reactions were stopped at intervals of time by tipping 50% (w/v) trichloroacetic acid from a side arm of the appropriate flask (Fig. 2). There was a rapid evolution of 1 mole of carbon dioxide/mole of glyoxylate within 5 min. and thereafter carbon dioxide continued to be liberated more slowly; this slower reaction was abolished when DPNH was present to reduce the C_3 intermediate to glycerate.

In the following experiments it was proved that the second slow reaction was not enzymic, contrary to the suggestion of Callely & Dagley (1959). Three series of tubes (8 mm. × 70 mm.) were set up to test three concentrations of cell extract. Each tube contained, in a volume of 0·2 ml., 0·75 μmole of potassium phosphate buffer, pH 7·2, and 0·76 μmole of sodium [1-^{14}C]glyoxylate (540 counts/min./0·02 ml. when plated on filter paper). Reactions were started by addition of 0·05 ml. of sonic extract of *Pseudomonas* (A) containing 1·2, 0·6 and 0·15 mg. of protein in each series respectively, and

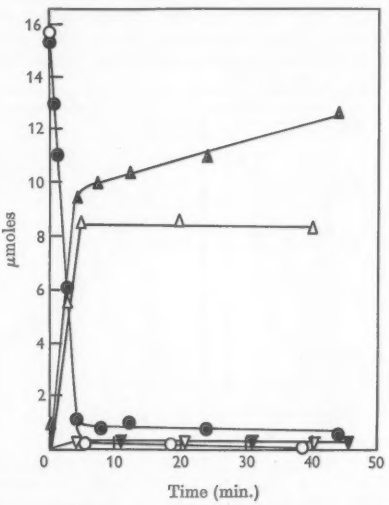

Fig. 2. Evolution of CO_2 from glyoxylate catalysed by crude extracts of glycine-grown *Pseudomonas* (A). The main compartments of double-armed Warburg flasks contained, in a total of 3 ml., 15 μmoles of potassium phosphate (pH 7·2) and 0·5 ml. of sonic extract (12 mg. of protein). Manometers were gassed with N_2 and equilibrated at 30°. Sodium glyoxylate (8 μmoles) with or without DPNH (5 μmoles) was added from side arm 1. Reactions were stopped by the addition of 0·2 ml. of 50% (w/v) trichloroacetic acid from side arm 2; the glyoxylate remaining and CO_2 evolved (μmoles) were then measured. DPNH added: △, CO_2 evolved; ○, glyoxylate remaining. No DPNH added: ▲, CO_2 evolved; ●, glyoxylate remaining. Controls with no glyoxylate added: ▽, DPNH added; ▼, no DPNH added.

the disappearance of glyoxylate with time was followed by chromatography, radioautography, elution and counting (Fig. 3*a*). These results were then used to study the effect of cell extract on the stability of the compound formed from glyoxylate. At intervals of time, each interval being measured from the point at which 90% of the initial glyoxylate had disappeared, 0·6 μmole of DPNH was added to each one of a series of reaction mixtures. In this way the C_3 intermediate was assayed by rapid conversion into [^{14}C]glycerate, which was identified by chromatography and counted. From Fig. 3*b* it appears that the rate of disappearance of intermediate, about 50% in 59 min. at 30°, was independent of the amount of extract present, and accordingly its decomposition was not enzymically catalysed. At 100° all the intermediate decomposed to glycolaldehyde in 1 min.; the stoicheiometry of the overall reaction agreed with the equation: 2 glyoxylate → glycolaldehyde + 2 CO_2 (Table 1).

Thiamine pyrophosphate and Mg^{2+} ions were cofactors for the enzymic decarboxylation of glyoxylate (Table 2) as shown also by Krakow *et al.* (1961) and Kornberg & Gotto (1961*a*). When DPNH was present in the reaction, 1 mole was oxidized and 1 mole of glycerate was formed with the disappearance of 2 moles of glyoxylate (Table 3*a* and *b*).

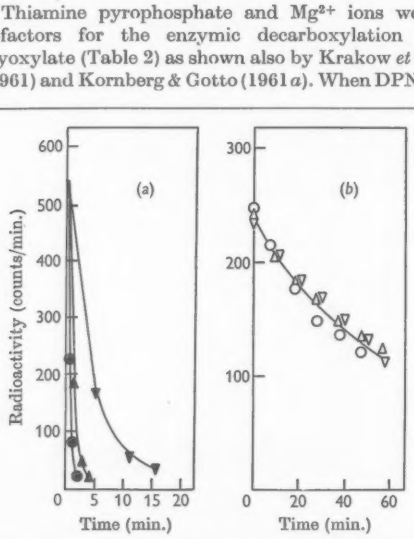

Fig. 3. Rate of decomposition of the C_3 compound formed from [1-^{14}C]glyoxylate, and its lack of dependence on cell-extract concentration. Reaction tubes (8 mm. × 70 mm.) contained, in 0·2 ml., 0·75 μmole of potassium phosphate (pH 7·2) and 0·76 μmole of sodium [1-^{14}C]glyoxylate (540 counts/min./0·02 ml. of sample counted). After equilibration at 30° amounts of cell extract were added containing: ○ ●, 1·2 mg. of protein; △ ▲, 0·6 mg. of protein; ▽ ▼, 0·15 mg. of protein. (*a*) Disappearance of glyoxylate at each extract concentration, determined by chromatography, radioautography, elution and counting of [1-^{14}C]glyoxylate. (*b*) Disappearance of C_3 compound, determined by conversion into [^{14}C]glycerate by addition of DPNH.

Reactions and properties of the C_3 compound

Krakow & Barkulis (1956) found that hydroxypyruvate was formed by decomposition of glyoxylate, but they showed later (Krakow *et al.* 1961) that it was not the initial product of the reaction; this, they suggested, was its isomer tartronic semialdehyde, which was converted into hydroxypyruvate by the acid used in deproteinizing reaction mixtures. Although we incubated such mixtures with acid solutions of 2,4-dinitrophenylhydrazine on many occasions we found no trace of the 2,4-

Table 1. *Stoicheiometry of the conversion of glyoxylate into glycolaldehyde*

Reaction mixtures contained, in 2·5 ml., 15 μmoles of potassium phosphate (pH 7·2), 2·5 μmoles of thiamine pyrophosphate, 2·5 μmoles of $MgCl_2$ and amounts of sodium glyoxylate and DPNH as stated. The reaction was started by addition of 0·5 ml. of dialysed cell extract (10 mg. of protein) and stopped after 15 min. by heating at 100° for 1 min.: this also converted the enzymic reaction product into glycolaldehyde. Residual glyoxylate was determined by the method of Friedemann & Haugen (1943) and glycolaldehyde as in the Materials and Methods section.

Glyoxylate added (μmoles)	Glyoxylate used (μmoles)	DPNH added (μmoles)	Glycol-aldehyde formed (μmoles)
7·9*	0	0	0·1
7·9	6·7	0	3·3
3·9	3·5	0	1·7
3·9	3·8	4	0·2

* Cell extract boiled before incubation.

Table 2. *Requirement for thiamine pyrophosphate and magnesium ions of glyoxylate carboligase present in cell extracts of glycine-grown* Pseudomonas (*A*)

Reaction mixtures contained, in 9·5 ml., 17·2 μmoles of sodium glyoxylate, 7·5 μmoles of potassium phosphate (pH 7·2), 5 μmoles of thiamine pyrophosphate (TPP) and 5 μmoles of $MgSO_4$. Reactions at 30° were started by adding 0·5 ml. of a Hughes press extract of cells dialysed for 24 hr. against phosphate buffer. After 15 min. protein was precipitated by the addition of 50% (w/v) trichloroacetic acid, and residual glyoxylate determined.

Tube no.	Contents	Glyoxylate (initial) (μmoles)	Glyoxylate (final) (μmoles)
1	Complete system	17·2	2·7
2	Enzyme omitted	17·2	17·1
3	TPP and $MgSO_4$ omitted	17·2	17·1
4	TPP omitted	17·2	12·8
5	$MgSO_4$ omitted	17·2	10·6

dinitrophenylhydrazone of hydroxypyruvate; therefore detectable non-enzymic conversion of the C_3 compound into hydroxypyruvate in the presence of acid did not occur in the conditions we used. DPNH and TPNH were rapidly oxidized in the presence of active glyoxylate carboligase (Fig. 4), the cofactors for which are thiamine pyrophosphate and Mg^{2+} ions (Table 2). Oxidation was faster, even at one-tenth of the enzyme concentration, when glyoxylate was first incubated with active extract to form the C_3 compound; hydroxypyruvate was reduced much more slowly than the intermediate. We confirmed the observation of Krakow *et al.* (1961) that some of the C_3 compound is formed after prolonged incubation of hydroxypyruvate with cell extract since the initial rate of oxidation of DPNH was thereby increased.

Kornberg & Gotto (1961*a*) have summarized evidence that the C_3 compound is tartronic semialdehyde. However, since no authentic sample of tartronic semialdehyde has yet been described we have recorded some additional properties of aqueous solutions obtained by enzymic decarboxylation of glyoxylate. On addition of 0·1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid to a dilute solution of C_3 compound, orange–red crystals separated after 15 min. at 30° and from its purple colour with alkali this appeared to be a bis-2,4-dinitrophenylhydrazone. At first, examination of its properties was hindered by the non-enzymic decomposition of C_3 compound to glycolaldehyde, which also forms a bis derivative with the reagent; however, these crystals separate after 1 hr. at 30°; glyoxal gives an immediate precipitate of the same bis-2,4-dinitrophenylhydrazone as glycolaldehyde.

Since the bis derivatives streaked across the papers, chromatography with various solvents was in general of no value except for the reverse-phase

Table 3. *Conversion of glyoxylate into glycerate by extracts of glycine-grown* Pseudomonas (*A*)

(a) Oxidation of reduced diphosphopyridine nucleotide by cell extracts and glyoxylate

Silica cuvettes contained, in 3 ml. of reaction mixture at 20°, 15 μmoles of potassium phosphate (pH 7·2), 0·39 μmole of DPNH, 1 μmole of thiamine pyrophosphate, 1 μmole of $MgCl_2$, 1 ml. of sonic extract (1 mg. of protein) and sodium glyoxylate. In control cuvettes DPNH was omitted. The amount of DPNH that reacted was calculated from the change in extinction at 340 mμ. The product of reaction was identified as glyceric acid.

Glyoxylate used (μmole)	DPNH oxidized (μmole)	DPNH oxidized / Glyoxylate used
0·20	0·097	0·48
0·40	0·21	0·51
0·56	0·28	0·50

(b) Stoicheiometry of the formation of [^{14}C]glycerate from [1-^{14}C]glyoxylate

Reaction tubes (8 mm. × 70 mm.) contained, in 0·2 ml., 0·75 μmole of potassium phosphate (pH 7·2), 0·76 μmole of sodium [1-^{14}C]glyoxylate and 0·6 μmole of DPNH. Reactions at 30° were started by the addition of 0·05 ml. of cell extract (1·2 mg. of protein); after 5 min. protein was removed and 0·05 ml. of liquid from each tube was applied to chromatograms. Glyoxylate and glycerate were assayed by radioautography, elution and counting.

Glyoxylate added (μmole)	Glyoxylate used (μmole)	DPNH added (μmole)	Glycerate formed (μmole)
0·76*	0	0·60	0
0·76	0·76	0·0	0·06
0·76	0·76	0·60	0·37

* Cell extract boiled.

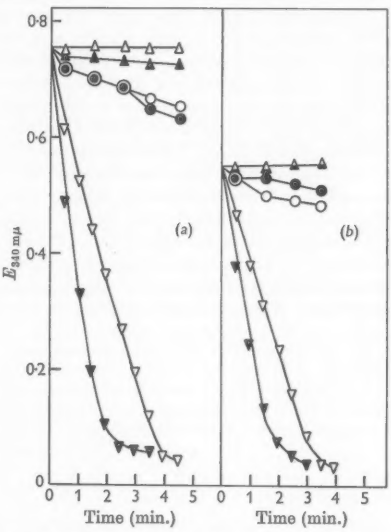

Fig. 4. Oxidation of reduced phosphopyridine nucleotides catalysed by sonic extracts of *Pseudomonas* (A) in the presence of glyoxylate or C_3 compound. (*a*), DPNH; (*b*), TPNH. In one experiment reaction mixtures contained, in 3 ml., 1 ml. of dialysed extract (1 mg. of protein) with 15 μmoles of potassium phosphate (pH 7·2), 0·5 μmole of thiamine pyrophosphate, 0·5 μmole of $MgCl_2$ and either 0·37 μmole of DPNH or 0·27 μmole of TPNH. Reactions were started by addition of: ▽, sodium glyoxylate (1·6 μmoles); ○, lithium hydroxypyruvate (0·8 μmole); or ●, glycolaldehyde (0·8 μmole). The decrease in extinction at 340 mμ was then measured. In control experiments extract (△) or glyoxylate (▲) was omitted. In another experiment the cell extract was diluted and 1 ml. (0·1 mg. of protein) preincubated for 40 min. at 20° with sodium glyoxylate (1·6 μmoles), thiamine pyrophosphate (0·5 μmole) and $MgCl_2$ (0·5 μmole). Reactions (▼) were started by addition of either 0·37 μmole of DPNH or 0·27 μmole of TPNH.

system of Meigh (1956) in which the derivatives ran as yellow spots, those of the C_3 compound and 2,4-dioxovaleric acid near the solvent front ($R_F > 0{\cdot}9$) and those of glycolaldehyde and the methyl ester of 2,4-dioxovaleric acid more slowly (R_F 0·4–0·5). Pure samples were obtained by elution with the upper layer of the solvent system of Meigh (1956) and their spectra in alkali plotted; maxima were at 550 mμ for the C_3 intermediate and 565 mμ for glycolaldehyde (Fig. 5). The solutions were respectively red–purple and deep purple. The behaviour of the bis derivative of the C_3 compound on chromatograms suggested the presence of a carboxyl group. In agreement, a derivative formed after treating [1-^{14}C]glyoxylate with cell extract retained radioactivity which ran with $R_F > 0{\cdot}9$, but when the enzyme product was first decarboxylated by heating to 100° for 1 min. the derivative was not radioactive, ran with R_F 0·5 and had a spectrum in alkali identical with that of the bis compound of glycolaldehyde. The bis-2,4-dinitrophenylhydrazone was much more stable than the C_3 compound from which it was formed and did not lose radioactivity in 2N-hydrochloric acid, 2% (w/v) potassium hydroxide in ethanol or when maintained at 100° for several hours. The bis derivative of glycolaldehyde was readily extracted by ethyl acetate from its solution in 1·5N-sodium hydroxide, but in accordance with its possession of a carboxyl group the derivative of the C_3 compound was extracted only after more prolonged shaking.

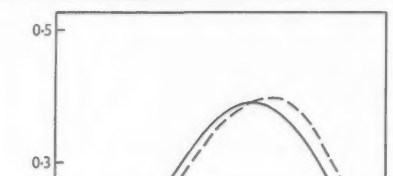

Fig. 5. Absorption spectra of the bis-2,4-dinitrophenylhydrazones of the C_3 compound and glycolaldehyde. The derivatives were purified by reverse-phase chromatography and eluted with chromatographic solvent. A clear area of the chromatogram was similarly treated to serve as a blank. Eluents were dried under vacuum and dissolved in 0·3% sodium ethylate, and their spectra recorded. – – –, Glycolaldehyde derivative; ——, C_3 compound derivative.

Sodium carbonate (10%, w/v) would not extract the C_3 derivative from its ethyl acetate solution, and by use of these solvents it was therefore possible to determine glyoxylate as its 2,4-dinitrophenylhydrazone in the presence of its decarboxylation products.

Enzymic conversion of glycerate into pyruvate

Extracts of *Pseudomonas* (A) grown with glycine catalysed a conversion of [^{14}C]glycerate into [^{14}C]-pyruvate dependent on the presence of ATP (Table 4). During the conversion two reaction intermediates were formed (Fig. 6) which gave colour reactions on chromatograms with a reagent specific for organic phosphates (Burrows, Grylls & Harrison, 1952). Since they were also converted almost completely into glyceric acid by calf-intestinal-mucosa phosphatase (L. Light and Co. Ltd.) it may be assumed that the compounds were phosphoglyceric acids (Table 5). When incubated with ADP, sonic extracts of *Pseudomonas* (A) converted phosphoenolpyruvate, 2-phosphoglyceric acid and 3-phosphoglyceric acid into pyruvate (Table 6). Similar results were obtained when ADP was replaced by ATP. The overall conversion of [1-^{14}C]glyoxylate into [^{14}C]pyruvate in the presence of DPNH and ATP was also shown (Fig. 6).

Malate synthetase

This enzyme (Wong & Ajl, 1956) could not be studied by using undialysed extracts since glyoxylate was rapidly removed from reaction mixtures by glyoxylate carboligase. After dialysis against 0·02M-phosphate buffer, pH 7·2, for 36 hr. at 4° a cell extract catalysed the conversion of sodium [1-^{14}C]glyoxylate into [^{14}C]malate when sodium acetate, ATP, coenzyme A and Mg^{2+} ions were present (Table 7). The yield of malate was unaffected by addition of reduced glutathione.

Table 4. *Conversion of glycerate into pyruvate by a cell extract of* Pseudomonas (*A*)

Reaction mixtures at 30° contained, in 0·2 ml., 0·75 μmole of [^{14}C]glycerate and 0·05 ml. of undialysed sonic extract (1·2 mg. of protein). The reaction was started by addition of 0·5 μmole of ATP and stopped after 30 min. by heating to 100°. The reaction product was converted into a 2,4-dinitrophenylhydrazone, and identified as the derivative of pyruvate by co-chromatography and radioautography. Spots were eluted, plated out and assayed for radioactivity in a gas-flow counter.

ATP added (μmole)	Radioactivity (counts/min.) Glycerate (initial)	Glycerate (final)	Pyruvate formed
0·5*	226	228	(2)
0	230	228	0
0·5	228	30	132

* Cell extract boiled before incubation.

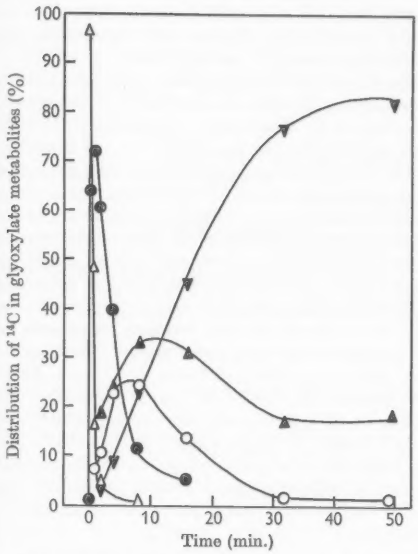

Fig. 6. Alteration with time of the percentage distribution of ^{14}C in the products formed by the action of a sonic extract of glycine-grown *Pseudomonas* (A) on sodium [1-^{14}C]glyoxylate. Reaction tubes contained, in 0·2 ml., 0·75 μmole of potassium phosphate (pH 7·2), 0·76 μmole of sodium [1-^{14}C]glyoxylate, 0·6 μmole of DPNH and 0·5 μmole of ATP. After equilibration at 30° reactions were started by the addition of 0·05 ml. of extract (1·2 mg. of protein). Glyoxylic acid (△), glyceric acid (●), and phosphoglyceric acid (○, ▲) were determined by direct counting of radioactive spots after chromatography as free acids. Pyruvate (▼) was determined after chromatography of its 2,4-dinitrophenylhydrazone.

Table 5. *Action of alkaline phosphatase on reaction intermediates in the conversion of glycerate into pyruvate*

Reaction mixtures for the preparation of these intermediates contained, in 0·25 ml., 0·75 μmole of potassium phosphate (pH 7·2), 0·6 μmole of DPNH, 0·5 μmole of ATP and 0·8 μmole of sodium [1-^{14}C]glyoxylate (1·25 μc). Reactions at 30° were started by the addition of 0·05 ml. of sonic extract (1·2 mg. of protein) and stopped after 8 min. by heating at 100° for 1 min. To 0·05 ml. of the mixture was added 0·05 ml. of a solution of 1 mg. of alkaline phosphatase (calf intestinal mucosa) in glycine–NaOH buffer, pH 9·5, containing 5 mM-magnesium acetate. After 30 min. at 30° protein was removed and the products were assayed by chromatography, radioautography and counting.

Alkaline phosphatase added	Reaction intermediates (% of total count)	Glycerate (% of total count)
Boiled	90	10
Active	8	92

Table 6. *Conversion of phosphoenolpyruvic acid, 2-phosphoglyceric acid and 3-phosphoglyceric acid into pyruvic acid by cell extracts*

Reaction mixtures at 30° contained, in 0·16 ml., 0·75 μmole of potassium phosphate (pH 7·2), 0·5 μmole of ADP and 0·4 μmole of substrate. The reactions were started by addition of 0·05 ml. of sonic extract of glycine-grown cells (1·2 mg. of protein) and stopped after 20 min. Pyruvate was identified as its 2,4-dinitrophenylhydrazone and estimated by the method of Friedemann & Haugen (1943).

Substrate added	ADP added (μmole)	Pyruvate formed (μmole)
Phosphoenolpyruvate	0·5*	0·04
	0	0·03
	0·5	0·30
2-Phosphoglycerate	0·5*	0·02
	0	0·03
	0·5	0·14
3-Phosphoglycerate	0·5*	0·04
	0	0·02
	0·5	0·20

* Cell extract boiled before incubation.

Table 7. *Malate synthetase in extracts of glycine-grown* Pseudomonas (*A*)

Reaction mixtures contained, in 0·35 ml., 0·75 μmole of potassium phosphate (pH 7·2), 0·76 μmole of sodium [1-^{14}C]glyoxylate, 1 μmole of $MgCl_2$, 1·6 μmoles of ATP, 1·6 μmoles of sodium acetate and 0·1 μmole of coenzyme A. Reactions at 30° were started by addition of 0·05 ml. of sonic extract (1·1 mg. of protein) that had been dialysed for 24 hr. against phosphate buffer (pH 7·2) and were stopped after 30 min. when protein was removed and solutions were chromatographed. Malate was located and counted directly with a Geiger–Müller end-window tube; its identity was confirmed by co-chromatography and radioautography.

Tube no.	Contents	Malate (% of total counts)
1	Complete system	55
2	Complete system*	6
3	Sodium acetate omitted	0
4	ATP omitted	0
5	Coenzyme A omitted	0
6	$MgCl_2$ omitted	11

* Cell extract boiled before incubation.

DISCUSSION

Campbell (1955) proved that glycine was oxidized by a species of *Pseudomonas* to carbon dioxide, ammonia and water with glyoxylic acid and formic acid as reaction intermediates. Cells grew in a medium containing, in addition to glycine, 1 g. of yeast extract/l. This concentration would support abundant growth of *Pseudomonas* (A) which, in our experiments, was supplied with

no source of carbon except glycine. It is possible that the enzyme system studied by Campbell (1955) operates for the removal of glycine, rather than for its utilization, by cells assimilating other nutrients. Our extracts differed chiefly from his in their failure to oxidize glyoxylate, which instead was vigorously decarboxylated. The significance of this reaction in the economy of the cell has already been discussed for bacteria utilizing as sole sources of carbon glycollate (Kornberg & Gotto, 1959), glycine (Callely & Dagley, 1959) and oxalate (Quayle & Keech, 1959). If intermediates of the tricarboxylic acid cycle are used for biosynthesis of cell constituents, ancillary reactions must operate for their replenishment when bacteria grow with C_2 compounds as sole source of carbon. When the C_2 compound is acetate or fatty acids that give rise entirely to acetyl-coenzyme A (Callely, Dagley & Hodgson, 1958), reactions occur by which glyoxylate is formed to enable the cycle to be replenished with malate, synthesized from acetyl-coenzyme A and glyoxylate (Kornberg & Krebs, 1957). When the C_2 acid contains more oxygen than acetate and gives rise to glyoxylate, enzymes convert some glyoxylate into the acetyl-coenzyme A needed for malate synthesis (Kornberg & Gotto, 1961*a*). Our experiments with whole cells, cell extracts and inhibitors suggest that the tricarboxylic acid cycle operates in *Pseudomonas* (A) grown with glycine as carbon source. The following enzymes which lie on the proposed pathway of conversion of glycine into acetyl-coenzyme A were present in extracts of these cells: glyoxylate carboligase, a reductase for conversion of the product of this reaction into glycerate ('tartronic semialdehyde reductase'), and enzymes for converting glycerate into pyruvate; and their cofactors were those found by other workers. Malate synthetase was also present. Cell suspensions converted glycine into pyruvate during a lag period before cell division began (Fig. 1), whereas serine dehydrase could not be detected in such cells. The proposed conversion of glycine into pyruvate, which would then give acetyl-coenzyme A, is as follows:

$$2\ \text{Glycine} + O_2 \rightarrow 2\ \text{glyoxylate} + 2NH_3 \quad \text{(i)}$$

$$2\ \text{Glyoxylate} \rightarrow [\text{tartronic semialdehyde}] + CO_2 \quad \text{(ii)}$$

$$[\text{Tartronic semialdehyde}] + \text{DPNH} + H^+ \rightarrow \text{glycerate} + \text{DPN}^+ \quad \text{(iii)}$$

$$\text{Glycerate} \rightarrow \text{pyruvate} + H_2O \quad \text{(iv)}$$

Sum:

$$2\ \text{Glycine} + O_2 + \text{DPNH} + H^+ \rightarrow \text{pyruvate} + 2NH_3 + CO_2 + H_2O + \text{DPN}^+ \quad \text{(v)}$$

The conversion of glycerate into pyruvate (reaction iv) is assumed to include well-established reactions of the Embden–Meyerhof scheme since we have shown (Fig. 6 and Tables 4–6) that phosphate esters of glyceric acid are involved.

The C_3 product of glyoxylate-carboligase action is assumed to be tartronic semialdehyde for reasons summarized by Kornberg & Gotto (1961*a*). Solutions at pH 7·2 when incubated for less than 1 hr. at 30° may contain appreciable amounts of its decomposition product, glycolaldehyde; this second decarboxylation is non-enzymic (Fig. 3*b*). The bis-2,4-dinitrophenylhydrazone is more stable than the tartronic semialdehyde from which it is formed, but samples may be contaminated with the derivative of glycolaldehyde which it resembles closely. The two bis derivatives can be well separated by reverse-phase chromatography, and when eluted give spectra in alkali differing by 15 mμ in the wavelengths of their absorption maxima. The behaviour of the bis derivative of glycolaldehyde formed from [1-^{14}C]glyoxylate is consistent with the presence of a carboxyl group in the bis-2,4-dinitrophenylhydrazone of tartronic semialdehyde; this is radioactive when produced from [1-^{14}C]-glyoxylate.

SUMMARY

1. When *Pseudomonas* (A) grown with glycine as sole carbon source was harvested after 20 hr. in the stationary phase and suspended in fresh medium, cell division was delayed for 4 hr. and pyruvate accumulated. Fluoroacetate and arsenite inhibited oxidation of glycine by these cells. They contained no serine dehydrase.

2. Extracts of cells grown with glycine catalysed the evolution of one molecule of carbon dioxide from two molecules of glyoxylate. This enzyme (glyoxylate carboligase) required thiamine pyrophosphate and magnesium ions for maximal activity. The product, presumed to be tartronic semialdehyde, decomposed spontaneously at pH 7·2 at the rate of 50 % in 50 min. to give glycolaldehyde.

3. The bis-2,4-dinitrophenylhydrazone of tartronic semialdehyde was separated from that of glycolaldehyde (glyoxal) by reverse-phase chromatography, and its absorption spectrum in alkali obtained.

4. Cell extracts contained an enzyme (tartronic semialdehyde reductase) that catalysed reduction of tartronic semialdehyde to glycerate. When coupled with glyoxylate carboligase, one molecule of glycerate was formed from two molecules of glyoxylate, and one molecule of reduced diphosphopyridine nucleotide was oxidized.

5. Cell extracts converted glycerate into pyruvate, phosphate esters of glyceric acid being formed as reaction intermediates. Malate synthetase was present in extracts.

6. From these results it appears that compounds of the tricarboxylic acid cycle are synthesized from glycine by a scheme similar to that suggested for another species of *Pseudomonas* grown with glycollate as carbon source (Kornberg & Gotto, 1961*a*).

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The Total Intracellular Concentration of Solutes in Yeast and Other Plant Cells and the Distensibility of the Plant-Cell Wall

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It is primarily intended to determine in yeast and other plant cells the total intracellular molar concentration of free solutes, including ion species, which may be conveniently stated as total intracellular molarity, the molarity of any single solute being the moles/l. of intracellular water (or kg. of such water without appreciable relative error).

In determining total intracellular molarities a variant of the cryoscopic method after breakage of the cell walls by freezing in liquid oxygen and thawing has been used in the experiments described, attention being given to the volume of the extracellular fluid as well as to the possibility of autolytic changes increasing the total molarity in the frozen and thawed mixture. Such changes occur rapidly even at 0° in certain animal tissues (Conway, Geoghegan & McCormack, 1955).

The conversion of freezing-point depression so determined into total molecular concentrations involves the use of a conversion factor which is here taken as 1·86° per unit molarity. This, applied in general, is no doubt approximate but sufficiently accurate for present purposes.

It is also necessary to know the volume of the extracellular water. For centrifuged yeast this has been dealt with in some detail by Conway & Downey (1950), and similar considerations have been applied here to plant tissues in general as described under Methods.

The total intracellular molarity so determined for the resting yeast has been compared with that obtained by summing up the intracellular concentrations of the major constituents as analytically determined.

Measurements have also been made on yeast fermenting under a variety of conditions. Other measurements have been made on potatoes, carrots and sugar-beet, the last-named being of particular interest as an example of a plant tissue which contains, in addition to the usual content of metabolites and electrolytes, an exceptionally high concentration of sucrose inside the cells. The distensibility of plant-cell walls has also been investigated by using varying external concentrations of a non-penetrating solute, and a coefficient of distensibility has been defined. This is easy to determine and may prove useful for comparative investigations.

METHODS

Chemical methods

Amino acids. These were determined on protein-free yeast extracts by (*a*) a modified Folin colorimeter method (Walters, 1937) at wavelength 470 mμ, and (*b*) a micro-modification of the Pope & Stevens (1939) method in which the final titration was made from a Conway burette. Close agreement was obtained with the two methods, indicating that the sulphur-containing amino acids were either absent or present in very small amounts in these extracts.

Total ether-extractable acids. Portions (10 ml.) of yeast suspension (1 part in 6 of tap water) were added to 5 ml. of 10 N-H_2SO_4 in a Kutscher–Steudel type of extractor (Krebs, Smyth & Evans, 1940). The high concentration of acid was used to ensure that the yeast cells would be rapidly ruptured. The extraction was continued for 9 hr. after which the ether extract was dried over anhydrous Na_2SO_4 to remove any mineral acid that might have been carried over during the extraction. After very thorough washing of the Na_2SO_4 with successive portions of dry ether, the combined ether extracts were evaporated to dryness, and the residue was dissolved in 10 ml. of hot water, cooled and titrated electrometrically.

Succinic acid. After the titration the neutral solution was usually transferred to a 25 ml. volumetric flask and made up to volume, and the succinic acid content determined with succinic dehydrogenase (Weil-Malherbe, 1937; Krebs *et al.* 1940).

Hydroxy acids (ether-soluble). These were determined by subtracting succinic acid from the total of ether-extractable acids. The succinic acid amounted to about 70% of the total, the remainder being mainly lactic acid, with some other acids in addition.

Citric acid. This was determined colorimetrically on the protein-free extract by a modified pentabromoacetone method with vanadic acid as oxidant (Weil-Malherbe & Bone, 1949).

Keto acid. The method used was a modification of the colorimetric method developed by Lu (1939). Details were as given by Conway & Brady (1950).

Ortho- and meta-phosphate. A trichloroacetic acid extract of centrifuged yeast cells was first prepared in the manner described by Juni, Kamer, Reiner & Spiegelman (1948), who used it before the fractionation of acid-soluble phosphate.

Inorganic phosphate was precipitated by $Mg(NO_3)_2$ at an alkaline pH (the mixture standing in the refrigerator for 2–3 hr.). Separation of the inorganic phosphate into ortho- and meta-phosphate fractions was effected as described (Juni *et al.* 1948). The phosphate in both fractions was determined by the method of Berenblum & Chain (1938).

Hexose esters. These were determined by the method of Conway & Hingerty (1946), proceeding through a precipitation with neutral alcohol of a sample of the trichloroacetic acid solution after the removal of ATP with barium acetate.

Sodium and potassium. These were determined by flame photometry in a Beckman model DU spectrophotometer with flame attachment. Samples of twice-washed yeast were boiled in 10–20 vol. of distilled water and diluted to give a convenient photometer reading. Before reading, all samples were centrifuged.

Calcium. Twice-washed yeast (1 g.) was boiled for 5 min. with about 5–6 ml. of water. The suspension was then allowed to cool and 2 ml. of 20% trichloroacetic acid added. The total volume was adjusted to 10 ml. with distilled water, the suspension centrifuged, and 5 ml. of the supernatant taken. This was neutralized with dil. NH_3 and the Ca precipitated on addition of 2 ml. of 4% ammonium oxalate. This precipitate was centrifuged, washed with dilute NH_3 solution and dissolved in 2 ml. of N-H_2SO_4, and the oxalic acid liberated was titrated against standard $KMnO_4$ solution.

Magnesium. This was determined by the titan-yellow method of Young & Gill (1951).

Calculation of concentration in the yeast-cell water. The concentrations were expressed first per/kg. of washed and centrifuged yeast. As the amount of cell water in 1 kg. of such yeast is 0·49 l. the concentration/kg. of the centrifuged yeast is multiplied by 1/0·49 = 2·04.

Freezing-point depressions. These were determined by the micro-cryoscopic method of Conway & McCormack (1953). With the apparatus employed, 0·25 ml. or less of tissue fluid was required. The apparatus had a sensitivity of 0·002° and successive readings for a given sample did not normally differ by more than 0·01°.

Baker's yeast (*Saccharomyces cerevisiae*) as supplied by the Cork Yeast factory was used. [This was also the source of the yeast used in the experiments of Conway & Downey (1950).]

Yeast samples for freezing-point measurements were prepared as follows: After the cells had been washed twice in about 20 times their volume of tap water and centrifuged (the second centrifuging being continued for 20 min. at 3000 rev./min.), the supernatant fluid was poured off and the yeast, contained in stoppered plastic tubes, was plunged into liquid O_2 for 6–8 min. The tubes were then removed from the liquid O_2, their contents allowed to thaw, and then immediately reimmersed for a second period of 6–8 min. and allowed to thaw once more. This procedure gave a fluid mixture on which freezing-point measurements could be readily carried out.

The vegetable tissues were cut into thin slices (less than 1 mm. thick) and placed in perforated metal containers

that could be completely immersed in liquid O_2. They were then twice frozen and thawed in a similar manner to the yeast cells, the tissue slices in their containers being kept in a closed vessel during the thawing process in order to avoid excessive condensation on them of atmospheric moisture. Immediately after the second thawing the tissue slices were quickly broken up by grinding them in a small mortar, the resulting material was rapidly centrifuged and the supernatant fluid introduced into the small glass tubes used in the freezing-point apparatus.

All samples for freezing-point determinations were stored frozen in stoppered tubes and were allowed to thaw out immediately before being placed in the apparatus for determining the freezing point.

Measurement of the cell-water volume

First, the total water in vegetable tissues or centrifuged cell species was determined by dry-weight measurements of weighed fresh tissue samples or samples of centrifuged cells from which the supernatant water had been removed. The water between the cells plus that in the cell wall external to the cell membrane (this total being termed throughout here 'extracellular water') was then determined in a similar manner to that adopted for centrifuged yeast by Conway & Downey (1950). Weighed amounts of the finely sliced tissue were immersed in solutions containing various concentrations of a non-penetrating reducing sugar (D-galactose for potato and carrot tissue, and L-arabinose for sugar-beet). The volume of the immersion fluid used was 1 l./kg. of tissue. About 1 hr. was allowed for equilibrium to be established and the concentration of the reducing sugar in the external fluid was determined by the method of Bertrand (Bertho & Grassman, 1938), the ratio of the initial sugar concentration to its concentration at equilibrium only being required. Control suspensions in which the tissues were immersed in distilled water indicated that the amount of reducing sugars lost by the tissues under the conditions of these experiments was quite negligible.

From the data obtained the volume of the intracellular water and related quantities may be derived as shown in the following section, after the symbols used in the calculations have been defined.

Symbols used in the calculations

Where 1 kg. of tissue is mentioned here this may be taken as applicable also to 1 kg. of washed and centrifuged yeast. All volumes are expressed as litres, and volumes and concentrations refer to 1 kg. of the washed tissue before the 1 hr. immersion:

C_0 Molar concentration of the non-penetrating sugar in the immersion fluid used.

C_e Molar concentration of the same sugar in the extracellular fluid, after the immersion procedure described above.

C_i Total molar concentration of constituents within the cells, which are non-diffusible with respect to the membrane.

C_{i0} The same as C_i but with zero concentration in the extracellular fluid.

V_{e0} Volume of extracellular water before the immersion period.

V_{ec} Volume of water coming from cells as the result of immersion in the solution of non-penetrating solute.

V_i Volume of intracellular water in equilibrium with C_e.

V_{i0} Volume of intracellular water when C_e is zero.

Since each litre of immersion solution will be diluted by the addition of V_{e0} plus V_{ec} volumes of water, then:

$$C_e = C_0/(1+V_{e0}+V_{ec}). \quad (1)$$

From this equation

$$\frac{C_0-C_e}{C_e} = V_{e0}+V_{ec}. \quad (2)$$

With centrifuged yeast it has been shown (Fig. 2 of Conway & Downey, 1950) that on plotting $(C_0-C_e)/C_e$ against C_e, a linear relation is obtained up to $C_e = 0{\cdot}7$ M. (The non-penetrating sugars used were L-arabinose and D-galactose and the symbol C_m then used has been changed here to C_e.) When extrapolated to zero external concentration, this line cuts the ordinate at a level indicating the volume of the extracellular fluid (V_{e0})/kg. of the original tissue or the washed and centrifuged yeast. The linear relation indicates that V_{ec} is proportional to the external concentration of the non-penetrating sugar (C_e).

Thus one may write:

$$\frac{V_{ec}}{C_e} = b, \quad (3)$$

where b is the slope of the line.

In this the water abstracted from 1 kg. of vegetable tissue or of centrifuged yeast is considered. For comparative purposes, it is more suitable to deal with the water abstracted from 1 l. of cell fluid, by different sugar concentrations outside, and, starting from the condition where the cells are at equilibrium with water only as the external fluid, one may write:

$$\frac{V_{ec}}{C_e V_{i0}} = \frac{b}{V_{i0}} = d, \quad (4)$$

where d is termed the 'coefficient of distensibility'. It may be defined as the volume of water abstracted from 1 l. of cell water, which had been in equilibrium with external water only, by unit concentration of a non-penetrating solute. Alternatively it may be defined as the gain of cell water per unit concentration of non-penetrating solute when this is replaced by water, the gain in volume being expressed per litre of final cell water.

The greater the value of d the greater the exchange of water in accordance with the above definitions, and hence the greater the distensibility of the cell wall. So far, d may be regarded as an empirical coefficient, but it is hoped to give elsewhere an extended account of its theoretical significance.

RESULTS

Freezing-point depressions of frozen and thawed yeast and total intracellular concentration. Twelve determinations by the method described above gave a mean value of $0{\cdot}680 \pm 0{\cdot}04°$ for the freezing-point depression of frozen and thawed resting yeast. Assuming a freezing-point depression of 1·86°/mole, this corresponds to a mean value of 0·366 M in the frozen and thawed mixture. From the data of Conway & Downey (1950) the total water in the centrifuged yeast is 0·79 l./kg. The total extracellular water (which includes the outer

region considered by Conway & Downey is 0·30 l./kg. The intracellular water (V_{i0}) is therefore 0·49 l./kg. and the molarity of the cell water (C_{i0}) is $0{\cdot}366 \times 0{\cdot}79/0{\cdot}49 = 0{\cdot}59$M. It was considered that this value for the total intracellular concentration of resting yeast might differ from the true value owing to the presence, in the fluid obtained from yeast frozen in liquid oxygen and allowed to thaw, of high protein concentrations that might have effects similar to those described by Kahn & Majer (1955).

This possibility was investigated as follows. The effect of storage in the cold (1–2°) on the freezing-point depression of frozen and thawed yeast tissue was first determined. In contrast with results previously reported for certain mammalian tissues (Conway, Geoghegan & McCormack, 1955), the freezing-point depression of yeast increased only very slowly under these conditions and no appreciable change could be detected over a period of 4–5 hr. Samples (2 ml.) of the fluid material obtained after freezing and thawing were then dialysed in the cold against an equal volume of distilled water, the conditions being such that equilibrium was attained in a comparatively short time (2–3 hr.). The total intracellular concentration calculated from the freezing-point depression of the diffusate at equilibrium could then be compared with that obtained from direct measurements on the same sample of frozen and thawed yeast.

The results of four experiments of this kind are shown in Table 1. Taking for each yeast sample the percentage difference between the calculated intracellular molarities from mixture and diffusate the mean figure with the latter procedure was $-1{\cdot}2\%$ less than for the mixture, the range being $+1{\cdot}8$ to $-3{\cdot}4$. Such differences come within the sampling error, and the results show that no appreciable effect on the freezing-point depression is produced by the protein concentration of the frozen and thawed mixture.

Effect of fermentation on the total intracellular concentration of yeast. In Table 2 the total intracellular concentrations of yeast that had been fermented under a variety of conditions are compared with the mean value found for the resting cells.

In the fermentation experiments 1 in 20 (w/v) of washed and centrifuged yeast was suspended in the external fluid. After fermentation for 5 hr. the suspension was centrifuged, and the yeast was washed twice with distilled water and centrifuged for 20 min. at 3000 rev./min. after the second washing. The freezing-point depression was then determined in the usual way. Data are given

Table 1. *Freezing-point depression of frozen and thawed resting baker's yeast, with calculated total intracellular molarity*

Similar observations with diffusates were obtained by dialysis (0°). The values of C_{i0} are expressed as moles/l. of intracellular fluid in equilibrium with external water.

	Freezing-point depression		Calculated total intracellular molarities		Difference of the intracellular molarity from the diffusate and the mixture
Sample	Frozen and thawed mixture	Diffusate corrected for the extra water used	From frozen and thawed mixture (C_{i0})	From diffusate (C_{i0})	(%)
1	0·715°	0·725°	0·620	0·630	+1·6
2	0·675	0·650	0·585	0·565	−3·4
3	0·620	0·635	0·540	0·550	+1·8
4	0·715	0·675	0·620	0·590	−4·9
Averages	0·681	0·671	0·591	0·584	−1·2

Table 2. *Total intracellular molarities of resting and fermented baker's yeast*

The figures in parentheses refer to numbers of experiments. The mean value of the freezing-point depression of resting yeast is given with the S.E.M. Example of calculation of intracellular molarity:

$$\text{For resting yeast } C_{i0} = \frac{0{\cdot}680}{1{\cdot}86} \times \frac{0{\cdot}786}{(0{\cdot}786 - 0{\cdot}30)} = 0{\cdot}59.$$

Yeast	Freezing-point depression	Total water content (l./kg.)	Intracellular molarities (C_{i0})
Resting	0·680 ± 0·040° (12)	0·786 (2)	0·59
Fermented, glucose (5%)	0·935 (2)	0·755 (2)	0·81
Fermented, glucose (5%) + K^+ (0·2M)	1·005 (2)	0·755 (2)	0·87
Fermented, glucose (5%) + Na^+ (0·2M)	1·065 (2)	0·752 (2)	0·92
Fermented, glucose (5%) + Mg^{2+} (0·2M)	1·025 (2)	0·772 (2)	0·88

in Table 2 for yeast fermented in 5% glucose and in 5% glucose containing 200 m-equiv./l. of Na^+, K^+ or Mg^{2+} ions. Na^+ and K^+ ions were added to the suspensions as citrate, and Mg^{2+} ions as acetate. The mean of the total intracellular molarities from the eight experiments with fermented yeast was $0{\cdot}87 \pm 0{\cdot}045$, or 48% higher than for resting yeast. In the two experiments with glucose alone without added electrolytes, the mean was 0·81 M, or 37% higher than for non-fermenting cells.

Total intracellular molarities for vegetable tissues. In addition to yeast, the other vegetable cells examined were contained in slices of potato, carrot and sugar-beet. The non-penetrating sugars chosen for measurement of the extracellular water, and distensibility coefficient, were D-galactose for potato and carrot and L-arabinose for sugar-beet. The fact that over the 60 min. period of immersion the sugar used did not enter the cells appreciably is shown by the data in Table 3. For centrifuged yeast and sugar-beet the value of $(C_0 - C_e)/C_e$ was followed from 10 min. up to 60 and 90 min. respectively. The sugar (0·12 M) was used in the immersion fluid. For the carrot and potato it was observed from 60 min. with the immersion fluid containing 1·0 M- and 0·5 M-D-galactose for the potato and 1·0 M for the carrot. No significant increase in the value of $(C_0 - C_e)/C_e$ occurs from 10 to 60 min. in the centrifuged yeast, nor from about 30 to 90 min. in the sugar-beet slices. Also no significant increase occurs with the carrot and potato slices from 60 to 120 min.

If the sugar were entering the cells one could write:

$$\frac{C_0 - C_e}{C_e} = V_{e0} + V_e + V_x. \qquad (2a)$$

This equation is similar to equation (2) except that an extra V_x value is introduced giving the extra 'space' into which the external sugar may be said to diffuse when it enters the cells. V_x would increase with the time, so that with the tissue slices of sugar-beet, carrot or potato of less than 1 mm. thickness a steady increase of V_x and hence of the value of $(C_0 - C_e)/C_e$ would occur. From Table 3 it is evident that no significant increase occurs over the experimental period. In Table 4 the intracellular molarities (C_{i0} values) for potato, carrot, sugar-beet and yeast are given, being calculated from the other values listed. An example of the calculation for the potato is given in the Table.

Table 3. *Measurement of extracellular water of tissues*

Tissues were incubated with the non-penetrating solutes given below. For further details see text. Each value of $(C_0 - C_e)/C_e$ is the average of three experiments.

Cell species	Substance used	Concn. used (M)	$(C_0 - C_e)/C_e$ after 10 min.	30 min.	60 min.	90 min.	120 min.
Baker's yeast (centrifuged)	L-Arabinose	0·12	0·31	0·30	0·27	—	—
Baker's yeast (centrifuged)	D-Galactose	0·12	0·31	0·29	0·35	—	—
Sugar-beet	L-Arabinose	0·12	0·25	0·32	0·32	0·33	—
Potato	D-Galactose	1·0	—	—	0·51	—	0·51
Potato	D-Galactose	0·5	—	—	0·31	—	0·31
Carrot	D-Galactose	1·0	—	—	0·43	—	0·43

Table 4. *Total and extracellular water of certain vegetable cells; also intracellular molarities*

The numbers in parentheses give the numbers of experiments; in all cases the figures for the single experiments were close to the mean values. Example of calculation of the intracellular molarity: taking the potato values, the molarity is calculated as

$$\frac{0{\cdot}635}{1{\cdot}86} \times \frac{0{\cdot}86}{(0{\cdot}86 - 0{\cdot}14)} = 0{\cdot}41.$$

The symbols used in the Table are defined under Methods.

Tissue or cell species	Freezing-point depression of frozen and thawed mixture	Total water (l./kg.) ($V_{e0} + V_{i0}$)	Extracellular water from Fig. 1 (l./kg.) (V_{e0})	Intracellular molarities C_{i0} (M)
Potato	0·635° (3)	0·86 (4)	0·14	0·41
Carrot	1·045 (3)	0·90 (7)	0·09	0·62
Yeast	0·68 (12)	0·79 (3)	0·30	0·59
Sugar-beet	1·54 (5)	0·77 (3)	0·32	1·42

Comparative distensibilities of plant-cell wall. A practical 'coefficient of distensibility' suitable for comparisons between various plant tissues or cells is given under Methods. As pointed out there the relative amount of cell water per kilogram of tissue will vary with varying extracellular water, and it is the litre of cell water that is suitably taken for comparison.

From the slopes of the lines in Fig. 1 the values of V_{ec}/C_e have been determined, and, applying equation (4), values of 'd' for the potato, the carrot, sugar-beet and yeast have been calculated and are given in Table 5.

Note on the V_{e0} *values.* The V_{e0} values for the plant tissues and centrifuged yeast are given by the points where the lines in Fig. 1 cut the ordinate. The data for centrifuged yeast were taken from the results of Conway & Downey (1950). The value of V_{e0} for yeast so found was 0·30 l./kg. This figure is largely made up of the volume of water between the yeast cells (0·22 l./kg.). There is in addition the water in the outer metabolic region of the cells into which various small molecular substances penetrate (Conway & Downey, 1950).

The value of 0·22 l./kg. was determined by the use of inulin solutions (the theoretical value for rigid spheres being 26 % of the total volume). This value was very recently confirmed in this Laboratory (J. Veličković, unpublished work). He was extending the investigations of Rasajski & Veličković (1957) and used both inulin and polyvinylpyrrolidone (mol.wt. 38 000) in a number of experiments.

The outer metabolic region, making up the remainder of the 0·30 l./kg., was identified with the cell wall for reasons detailed in the paper of Conway & Downey (1950).

For the V_{e0} value with fermenting yeast no change need be considered, the amount of budding during the 5 hr. of fermentation, under the conditions, making no appreciable change in the volume of water between the centrifuged cells.

Comparison between the total intracellular concentration as determined cryoscopically and analytically in yeast. The total intracellular molarity was found to be 0·59 M in resting, washed yeast. The analytical data in Table 6 show that the sum of the known concentrations of intracellular substances in the yeast cell, of quantitative significance for this purpose, is 378 mM apart from the amino acids. If the amino acid nitrogen were all present in free monoamino acids the amount would be 224 mM of a total of 602 mM. It is very probable at the same time that the figure of 224 mM is somewhat in excess of the true figure through some of the acids being present in di- or tri-peptide form, as well as a proportion existing as the diamino acids. It is also very probable that much of the magnesium and calcium content is not free in the cell water.

It may be concluded, however, that the analytical results with respect to the composition of yeast indicate a total intracellular molarity of a similar order to that found by cryoscopic measurements.

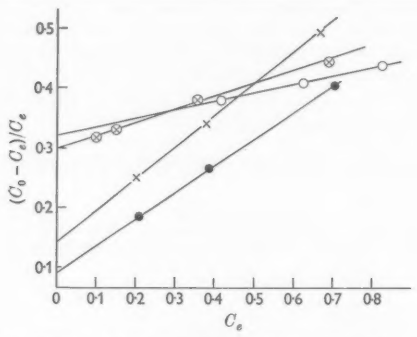

Fig. 1. Values of the external concentration (C_e) of the non-penetrating sugar plotted against the corresponding values of $(C_0 - C_e)/C_e = (V_{e0} + V_{ec})$, the symbols being defined under Methods. The data for centrifuged baker's yeast have been taken from the results of Conway & Downey (1950). ×, Potato; ⊗, yeast; ○, sugar-beet; ●, carrot.

Table 5. *Distensibility coefficients for certain vegetable cells*

The values of V_{i0} were obtained from the values of $(V_{e0} + V_{i0})$ and of V_{e0} in Table 4. The ratios V_{ec}/C_e were obtained from the slopes of the lines in Fig. 1. The values of C_{i0} (intracellular molarities) are given from Table 4 for comparison with the 'd' values.

Tissue or cell species	Intracellular water (V_{i0})	Ratio of water abstracted from cells divided by $C_e = (V_{ec}/C_e)$	Distensibility coefficients (d or $V_{ec}/C_e V_{i0}$)	Intracellular molarities (C_{i0})
Potato	0·72	0·531	0·74	0·41
Carrot	0·81	0·440	0·55	0·62
Yeast				
Resting	0·49	0·214	0·44	0·59
Fermented	—	—	—	0·92
Sugar-beet	0·45	0·147	0·33	1·42

Table 6. *Approximate total intracellular molarity of resting baker's yeast as determined by chemical analyses*

(B) indicates that such data were received from Professor T. Brady, University College, Cork (personal communication).

Substance	m-moles/kg. of resting yeast	No. of observations	Range observed	m-moles/l. of cell water
Potassium	128	34	102–148	261·8
Amino acids (B)	110	3	—	224·0
Orthophosphate	13·2	5	10·8–15·0	26·9
Succinic acid (B)	13·2	3	—	26·9
Magnesium	7·1	8	7·4–8·7	14·5
Hydroxy acids (ether-soluble) (B)	5·2	3	—	10·6
Hexose esters	4·7	5	4·4–5·4	9·6
Calcium	4·7	8	4·0–5·1	9·6
Citric acid (B)	3·5	3	—	7·1
Sodium	2·7	8	2·0–3·3	5·5
Metaphosphate	2·6	5	1·3–3·5	5·3
Keto acid (B)	0·4	3	—	0·8

An intracellular molarity of 0·59 M corresponds to an internal osmotic pressure of 13 atm., but for yeast fermenting in the presence of external Na^+ ions the intracellular molarity was found to be 0·92 M, corresponding approximately to 21 atm. For a sugar-beet sample with high sugar content the intracellular molarity was 1·42 M, corresponding to 32 atm. of osmotic pressure.

Relation between the difference in molarities on each side of the cell wall and the volume of the intracellular water. This relation is derived for yeast from the data given in Table 3 of the paper by Conway & Downey (1950).

If one takes, for example, the value of 0·693 M (mean for D-galactose and L-arabinose experiments) for the external solution when 1 kg. of centrifuged yeast is mixed with 1 l. of the sugar solution, then the value of C_0/C_e is 1·445 so that $(C_0-C_e)/C_e = 0{\cdot}445$. As given under Methods this is equal to $V_{e0}+V_{ec}$. Since V_{e0} is 0·30 (from Fig. 1) V_{ec} is $(0{\cdot}445-0{\cdot}30)$ or 0·145 when there is an external C_e of 0·693 M. As the value for V_{i0} or intracellular water for resting yeast in equilibrium with external water is 0·49 l./kg. the value of V_i in equilibrium with 0·693 M externally is $(0{\cdot}49-0{\cdot}145)$ or 0·345 l. The intracellular molarity is then $0{\cdot}49/(0{\cdot}345\times 0{\cdot}59\,\text{M}) = 0{\cdot}838\,\text{M}$, 0·59 M being the intracellular molarity in equilibrium with water. Proceeding in this way with the other figures in Table 3 of the paper of Conway & Downey (1950), it appears that a linear relation exists between (C_i-C_e) and V_i as shown in Fig. 2. This relation is given by the equation:

$$V_i = (C_i-C_e)\times 0{\cdot}34+0{\cdot}295,$$

or

$$(C_i-C_e)\times 0{\cdot}34 = V_i-0{\cdot}295. \qquad (5)$$

Thus when $C_i = C_e$, $V_i = 0{\cdot}295$, and it is only above this intracellular water volume that the cell wall becomes distended or develops tension, the increase in volume being linearly related to the difference in molarities (or resulting difference in osmotic pressure) of the solutions on each side of the cell wall.

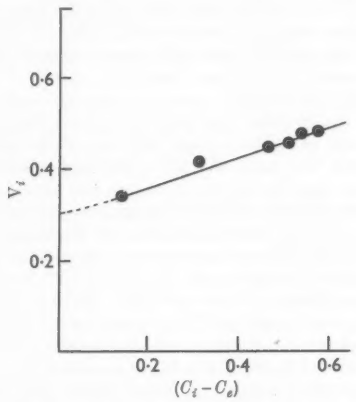

Fig. 2. For baker's yeast, and the non-penetrating sugars D-galactose and L-arabinose the Figure gives the values of (C_i-C_e) or the difference between the intracellular and extracellular molarities, against the values of V_i, the volume of the intracellular fluid (the symbols are defined in Methods). The original data are taken from Table 3 of Conway & Downey (1950).

DISCUSSION

The reality of the high intracellular pressure in plant cells, which even in resting yeast amounts to about 12 atm., is supported by three different kinds of evidence shown here. Thus the cryoscopic data

after bursting the cells by freezing and thawing are of the expected order and agree with the results obtained from the diffusate from this mixture. The total intracellular concentration of solutes in yeast as determined from the sum of the analytical data is in agreement with that indicated by the cryoscopic results; also, there is the fact that from zero to high external concentrations (e.g. to about 0·5M) of a solute to which the cells are impermeable, there is a practically linear relation between the external concentration and the cellular water loss. This is in accordance with a high internal pressure associated with water or dilute solutions outside.

There is in addition to such evidence the experiments of Eddy & Williamson (1957) using the observation of Giaja (1922) that the gut juice of the snail *Helix pomatia* dissolved the cell wall of whole yeast cells. They were able to prepare protoplasts or cells without cell walls which were stable for several hours when suspended in 0·5M-rhamnose. In sugar solutions greater than 0·5M the protoplasts contracted, and with lower concentrations they tended to swell and eventually burst.

Such observations accorded with the evidence given above that the internal concentration of solutes in the resting yeast cells was of the order of 0·5–0·6M. This work of Eddy & Williamson followed the new approach to the structure and functions of the cell surface of certain bacteria as revealed by Weibull (1953), who showed that, in the presence of sucrose, lysozymes dissolved the cell wall, leaving the protoplast essentially intact.

Distensibility of the cell wall. If the classical concept of elasticity and Young's modulus be applied to the cell wall, there arises the difficulty that the thickness of the wall alters with change in volume of the cell; also the thickness of the wall is not easy to determine with exactness. It would seem useful to have a more practical measure of cellular distensibility, such as is given by equation (4).

The results in Table 5 giving the d values of equation (4) for the potato, carrot, yeast and sugar-beet suggest that the total intracellular concentration goes in inverse proportion to the distensibility coefficients, though many more results would be required to test such a hypothesis.

The following comparison may be useful in clarifying the nature of the changes in intracellular volume as the vegetable cells or tissue slices containing them are immersed in solutions of a non-penetrating solute.

One may suppose a porous cylindrical vessel, with a semi-permeable membrane in the wall, and containing a certain concentration of a solute. Surrounding the cylinder is another solution. A piston fits over the surface of the inner fluid closely enough to prevent the passage of fluid between it and the cylinder wall, but otherwise free to move. On entrance of water into the cylinder the solution therein moves the piston up against a spring. When the molarities of the solutions outside and inside the cylindrical vessel are equal, the spring is considered fully extended and exerts no force on the piston. If the external solution is replaced by a weaker solution, water enters the cylinder and the piston presses up against the spring.

Here the compression of the spring is analogous to the distension of the cell walls, and the volume present when the molarities are equal inside and outside is analogous to the 0·295 in equation (5). When the molarity outside is less than inside, the volume of water that enters the cylinder is proportional to the difference between the internal and external molarities at equilibrium.

SUMMARY

1. By means of a cryoscopic method the intracellular molarities of baker's yeast, potato, carrot, and sugar-beet were found to be 0·59 (rising on fermentation to over 0·80), 0·41, 0·62 and 1·42M respectively.

2. The total intracellular molarity of resting yeast from the analysis of its various constituents was of the same order as that determined cryoscopically.

3. A 'coefficient of distensibility' for plant cells has been defined as the volume of intracellular fluid removed from the cells per unit concentration of an external non-penetrating solute and per unit volume of intracellular fluid. It is expressed as V_{ec}/C_eV_{i0}, the symbols being defined in the text. The 'coefficient of distensibility' or 'd' value is relatively easy to determine, as a linear relation has been found between V_{ec} and C_e up to high values of C_e, the external concentration. The values of the 'coefficient' for the potato, carrot, resting baker's yeast and sugar-beet were found to be 0·74, 0·55, 0·44 and 0·33 respectively.

4. The distension of the resting yeast cell, expressed as the volume of the intracellular fluid, gives a linear relation when plotted against the value of $(C_i - C_e)$. Here C_i and C_e are the total intracellular molarities and that of an external non-penetrating solute respectively. Extrapolating the line to zero value of $(C_i - C_e)$ it cuts the ordinate at 0·295 l. of intracellular fluid/kg. of centrifuged yeast.

Our thanks are due to Professor T. G. Brady, University College, Cork, for various analytical data, and also to the National Institute of Health, Washington, and the Research Department, American Air Force, for grants-in-aid.

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Studies in Immunochemistry

20. THE ACTION OF PAPAIN AND FICIN ON BLOOD-GROUP-SPECIFIC SUBSTANCES*

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Studies on the composition and structure of the human blood-group-specific substances, which are macromolecules containing carbohydrate units firmly bound to amino acid residues, have been largely concerned with the carbohydrate components, and the results have contributed much to our understanding of the part played by these structures in the characteristic serological reactions of the specific substances.

The nature and form of the amino acid-containing moiety of the materials has, however, been somewhat neglected and relatively little is known about this component except that it contains a remarkably high content of proline and of the hydroxy amino acids threonine and serine.

In earlier studies on the serologically specific materials isolated from the mucins which occur in human secretions and tissue extracts, proteolytic enzymes were employed to remove protein in the belief that the amino acids present constituted an impurity. Although much extraneous protein could be removed by proteolysis from the native mucins or tissue digests early in the isolation procedure, nevertheless it was found that the final product always contained amino acids. It is now known that amino acids comprise about 15% by weight of all highly purified human blood-group substances, irrespective of their specificity within the ABO and 'Lewis' blood-group systems.

Until recently, it was generally believed that no changes were induced in the materials by digestion with proteolytic enzymes, but a few early observations (Jorpes & Norlin, 1933; Jorpes, 1934; Landsteiner & Harte, 1940) suggested that a modification, or even a destruction of the serological properties of the substances, occurred. However, the changes observed were not further investigated.

In an attempt to obtain the blood-group-specific mucopolysaccharides from human secretions by a method which did not involve the use of phenol (Morgan & King, 1943), native secretions were incubated with ficin at pH 7 (Lawton, McLoughlin & Morgan, 1956). The materials recovered had a much reduced viscosity compared with the mucopolysaccharides isolated from the same native secretions by extraction with phenol, and other properties, such as solubility in 90% phenol and behaviour in the ultracentrifuge, were also changed. There was, at that time, no firm evidence to indicate that digestion with ficin decreased serological activity, but subsequently Pusztai & Morgan (1958) showed that treatment of the materials with ficin or papain lowered their capacity to neutralize the appropriate antibody in haemagglutination-inhibition tests.

* Part 19: Pusztai & Morgan (1961*b*).

These findings prompted a more detailed study of the action of crystalline ficin and papain on the serological and physical properties of highly purified mucopolysaccharides to be undertaken, and the results of the action of these proteolytic enzymes on some of the specific materials are now given.

EXPERIMENTAL

Blood-group-specific substances. The preparations of blood-group-specific mucopolysaccharides used were isolated from pseudomucinous ovarian-cyst fluids. The materials were obtained from 'secretors' (cf. Race & Sanger, 1958) belonging to each of the blood groups A_1, B, O, A_1B and A_2B, and from 'non-secretor' individuals. Material from the last group possessed the serological character known as Le^a (cf. Grubb & Morgan, 1949; Annison & Morgan, 1952; Race & Sanger, 1958); the mucopolysaccharide itself had only Le^a activity and was devoid of A, B and H specificity.

The serologically active group-specific mucopolysaccharides were isolated and purified as described by Morgan & King (1943) and Gibbons, Morgan & Gibbons (1955) and were insoluble in 90% (w/w) phenol and soluble in water. In most instances the materials were further purified by treatment with saturated $(NH_4)_2SO_4$ at 55–60° for a few minutes as described by Pusztai & Morgan (1961*b*). The mucopolysaccharide insoluble in saturated $(NH_4)_2SO_4$ under these conditions was repeatedly fractionated from solution in water by the addition of ethanol and the material precipitating between concentrations close to 48% and 55% ethanol was used for the experiments described below.

Enzymes. Twice- or thrice-crystallized papain and ficin were obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A., or from the Sigma Chemical Co., St Louis, Mo., U.S.A. The enzymes were suitably activated with 2,3-dimercaptopropanol (BAL) immediately before use. Crystalline chymotrypsin, trypsin and pepsin were obtained from The Armour Laboratories.

The proteolytic activity was estimated by the method of Anson (1938). The enzyme, activated with NaCN, was mixed with alkali-denatured bovine haemoglobin in phosphate buffer, pH 7·5, and incubated at 37° for exactly 10 min.; an equal volume of 10% trichloroacetic acid was added and the protein precipitate spun off after the mixture had stood for 15 min. at 1–3°. The extinctions of the supernatant fluids were read at 280 mμ in a Hilger quartz spectrophotometer with an optical path of 1 cm.

The proteolytic activity of the enzymes was also estimated at pH 5 with a synthetic substrate, *N*-benzoyl-arginine amide, prepared by the method of Grassmann & Heyde (1929) (cf. Kimmel & Smith, 1957). The rate of hydrolysis was followed by titration with ethanolic KOH of the carboxyl groups formed. Peptic activity was determined with acid haemoglobin substrate at pH 1·6, according to the method of Anson (1938).

Enzymic hydrolysis. A 1% (w/v) solution (1 ml.) of the serologically specific mucopolysaccharide in either 0·1 M-citrate–phosphate buffer (pH 4·0) or in 0·05 M-phosphate buffer (pH 7·6), each containing 10^{-3} mM-BAL, was mixed with different amounts of the enzyme dissolved in the same buffer (1 ml.), which also contained 10^{-3} mM-BAL. The enzyme–substrate mixtures were incubated at 37°. Toluene was added as preservative.

Viscosity measurements. The viscosity changes induced in the mucopolysaccharides by the enzymes were determined as follows. The mucopolysaccharide, dissolved (1–2%, w/v, soln.) in citrate–phosphate buffer containing 10^{-3} mM-BAL, was placed (1 ml.) in an Ostwald-type viscosimeter (flow time with water 120 sec. at 20°) and kept at 0° in a large bath containing crushed ice and water. Known amounts of activated ficin or papain dissolved in buffer were added and the mixture was drawn up repeatedly into the viscosimeter and allowed to flow down under gravity at 0°, the flow time being recorded. The values obtained remained unchanged for several hours. The viscosimeter was then transferred to a large, well-stirred water bath at 37° for a known time, during which period the contents were drawn up and down several times. The viscosimeter was then returned to the ice–water bath and, after an interval of several minutes, the flow time was again determined. This process was repeated at predetermined intervals until the enzyme–substrate mixtures had been in contact at 37° for a total of about 24 hr.

Measurement of pH *change.* The changes in H^+ ion concentration during digestion of the blood-group substances with enzyme were followed electrometrically, a method similar to that described by Jacobsen & Leonis (1949–51) being used. The pH measurements were made with a direct-reading pH meter (Electronic Instruments Ltd.).

Consumption of alkali during enzymic hydrolysis. Thoroughly dried group substance was dissolved in water containing BAL and the solution at 37° was adjusted to pH 7·00 with NaOH. The solution usually remained at this pH, but, if necessary, the mixture was readjusted to pH 7·00. The enzyme, dissolved in water and activated with BAL, was likewise adjusted to pH 7·00 at 37° and the pH of the solutions was finally checked immediately before mixing. The mixed solutions were kept at 37° and alkali (0·01 N-NaOH) was added to the thoroughly but gently stirred solution. Throughout the incubation the solution was maintained at pH 7·00. Observations were continued for at least 30 min. after the last addition of alkali was needed. Two control experiments were also included: one with heat-inactivated (30 min. at 100°) enzyme and substrate, and another with active enzyme, but without substrate. The consumption of alkali in each instance was recorded.

Serological examination. The specific serological activity of the materials was determined by haemagglutination-inhibition tests as described by Morgan & King (1943).

RESULTS

The change in the serological activity of each of the group-specific mucopolysaccharides induced by the action of ficin or papain shows that either enzyme, acting at pH 4·0 or 7·6, brings about considerable inactivation of each substance irrespective of its specificity (Table 1), and that the extent of the serological inactivation by either enzyme is the same within the sensitivity of the test, but is greater at pH 4·0 than at pH 7·6. The inactivation is limited in amount and, in spite of prolonged digestion, each substance retains a few

Table 1. *Changes in the serological activity of blood-group-specific mucopolysaccharides digested with ficin or papain*

Group A substance was incubated with 1% (w/w) of enzyme and group B and H substances with 5% (w/w) of enzyme. Materials were dissolved in 0·1 M-citrate–phosphate buffer (pH 4·0) or in 0·05 M-phosphate (pH 7·6). Enzymes were activated with 10^{-3} mM-dimercaptopropanol.

		pH 4·0		pH 7·6	
Group specificity	Enzyme	Time of digestion (hr.)	Serological activity* (μg.)	Time of digestion (hr.)	Serological activity* (μg.)
A	Ficin	0	0·8	0	0·8
		0·5	6	0·5	6
		1·0	12	1·0	12
		3·0	25	3·0	12
		24·0	50	24·0	12
B	Ficin	0	0·4	0	0·4
		20	6	20	3
	Papain	0	0·4	0	0·4
		20	6	20	3
H	Ficin	0	0·8	0	0·8
		16	6	20	3
	Papain	0	0·8	0	0·8
		16	6	16	3
Le^a	Ficin	0	0·01	0	0·01
		20	0·08	20	0·04

* Minimum amount of material giving complete inhibition of haemagglutination.

per cent of its original activity. Incubation for periods in excess of 24 hr. or the addition of further quantities of enzyme do not reduce the activity further. Incubation of the substrates with heat-denatured (30 min. at 100°) enzyme for prolonged periods, with and without BAL etc., caused no serological inactivation.

Activators and inhibitors

The effect of different activators, which included reducing and metal-chelating substances, on the capacity of the enzymes to inactivate the group substances, and the effect of *p*-chloromercuribenzoic acid, known (cf. Kimmel & Smith, 1957) to abolish the proteolytic activity of ficin and papain, were also examined. Crystalline ficin was found (Pusztai, 1961) to contain 0·8 mole of reactive SH group/26 000 molecular weight on titration with *p*-chloromercuribenzoate (Boyer, 1954). The corresponding data used for crystalline papain are those of Finkle & Smith (1958).

The results (Table 2) show clearly that BAL and potassium cyanide are good activators, whereas *p*-chloromercuribenzoic acid completely inhibits the action of the enzymes on a preparation of A mucopolysaccharide. The inhibition by *p*-chloromercuribenzoate is abolished by dialysing the inhibited enzyme against water containing BAL, or by adding a large excess of this reagent to the inactive *p*-chloromercuribenzoate–enzyme complex. Similar results were obtained with H substance.

The dependence of the extent of the serological inactivation of a specific substance on the amount of activated enzyme present is shown by the results given in Table 3, which indicate that the inactivating enzyme is the main proteolytic component in the crystalline ficin or papain used.

The action of other enzyme preparations on H-specific mucopolysaccharide show (Table 4) that lysozyme, trypsin, plasmin, pepsin, chymotrypsin and a mould protease were without effect on the activity. The other specific substances behaved similarly.

The known inactivation of the specific substances by certain carbohydrase preparations obtained from micro-organisms (Schiff, 1939; cf. Kabat, 1956), and the specific inhibition of the enzymic destruction of the serological activity by relatively large amounts of simple carbohydrates (Watkins & Morgan, 1955), prompted us to determine, by the procedure described by these workers, whether simple sugars inhibited the inactivation by ficin or papain; we found no inhibition.

The preparations of ficin and papain used failed to liberate reducing sugars from α- and β-methyl-galactopyranosides, or from β-methyl-*N*-acetyl-glucosaminide.

Table 2. *Effect of dimercaptopropanol, potassium cyanide and* p*-chloromercuribenzoic acid on the serological inactivation of group A mucopolysaccharide by ficin or papain*

Group A substance in buffer (1 ml. of 1% soln.) wa added to 0·5 mg. of enzyme with or without activator or inhibitor. The mixtures were incubated at 37° for 16 hr. and titrated in haemagglutination-inhibition tests. PCMB, *p*-Chloromercuribenzoic acid.

pH of digestion mixture	Enzyme	Activator or inhibitor	Serological activity§ (μg.)
4·0	—	—	0·8
4·0	Ficin*	—	12·5
4·0	Papain*	—	12·5
4·0	—	10^{-3} mM-BAL	0·8
7·6	—	10^{-3} mM-BAL	0·8
7·6	—	10^{-3} mM-KCN	0·8
4·0	Papain	10^{-3} mM-BAL	50
7·6	Papain	10^{-3} mM-BAL	25
4·0	Ficin	10^{-3} mM-BAL	50
7·6	Ficin	10^{-3} mM-BAL	50
7·6	Papain	10^{-3} mM-KCN	50
7·6	Ficin	10^{-3} mM-KCN	50
4·0	Ficin	PCMB†	1·6
4·0	Ficin	PCMB‡	6·4
4·0	Ficin	PCMB‡ and 10^{-3} mM-BAL	50
4·0	Papain	PCMB†	1·6
4·0	Papain	PCMB‡	6·4
4·0	Papain	PCMB‡ and 10^{-3} mM-BAL	50

* Crude enzyme.
† Enzyme incubated with 10 times its quantity of PCMB (SH equiv. wt.) for 15 min. at 37° before adding to substrate.
‡ Enzyme incubated with equiv. wt. of PCMB for 15 min. at 37°.
§ Minimum amount of group substance giving complete inhibition of haemagglutination.

Table 3. *Dependence of the extent of serological inactivation of A substance on the enzyme concentration*

Substrate (0·5 ml. of 1% soln.) in citrate–phosphate buffer (pH 4) containing 10^{-3} mM-BAL and varying amounts of (*a*) ficin and (*b*) papain was used. Incubation, 16 hr. at 37°. Toluene was added as preservative.

Substrate:enzyme ratio	Serological activity* (μg.)	
	a	*b*
No enzyme	0·8	0·8
10 000:1	1·6	1·6
1 000:1	3·2	6·4
100:1	25	25
10:1	50	50

* Minimum amount needed to inhibit the test dose of haemagglutinin.

Changes in viscosity of mucopolysaccharide solutions induced by ficin and papain

The blood-group-specific substances have relatively high viscosities and experiments were made to determine whether, during serological inactivation of these materials by ficin or papain, there was a concomitant change in viscosity. The procedure for following the loss in viscosity is described in the Experimental section. Results obtained in a typical experiment with H substance (no. 299) are plotted in Fig. 1, where the relative viscosity of the material is seen to be reduced by activated papain from 3·00 to 1·93, whereas in the control experiment, with heat-inactivated enzyme, the viscosity value remained almost unchanged. When the enzyme:substrate ratio was 1:400, the viscosity, after a rapid initial fall, declined only very slowly, presumably because of a considerable decrease of active enzyme in the system. Additional enzyme, to make an enzyme:substrate ratio of 1:100, added at this time lowered the viscosity further. The viscosity remained constant at about 1·9 after the addition of a further 0·4 mg. of active enzyme. The addition of ficin after the changes brought about by papain were complete caused no further fall in viscosity. The dependence of the rate and extent of viscosity reduction on the enzyme:substrate ratio is shown (Fig. 2) for incubation of an Le^a-specific mucopolysaccharide with papain to give enzyme:substrate ratios which varied from 1:10 000 to 1:20. A heated enzyme control in a 1:20 enzyme–substrate mixture was included. Similar experiments were made with ficin and the other specific mucopolysaccharides as substrates. The viscosity fall is strongly dependent on the enzyme:substrate ratio, but a change in the ratio to less than 1:20 had no influence on the viscosity value finally obtained.

The activity of papain after incubation with mucopolysaccharide (enzyme:substrate ratio 1:20) and without substrate was measured by its action on haemoglobin, as described by Anson (1938), and

revealed that the enzyme is quite extensively inactivated in 16 hr. at 37°, whereas the proteolytic activities of crystalline pepsin at pH 1·8 and of a crystalline mould protease were only slightly reduced (Table 5). These results show that small amounts of enzyme are inactivated and fail to bring about, after a longer time of action, the fall in viscosity induced by larger amounts of enzyme acting for a shorter time. The action of ficin and papain on A mucopolysaccharide shows the characteristic pH dependence of the enzyme action (Figs. 3 and 4). The fall in viscosity is expressed in per cent of the original viscosity and is plotted against the pH of the reaction mixture. The Figures contain a family of curves, each one of which is obtained by using viscosity values obtained after various time intervals (10, 20, 60 min. etc.) for each reaction mixture. It is evident that there is a characteristic pH optimum near pH 6 for both enzymes, when the viscosity values reached after the first 10 min. interval (the initial reaction velocity) are used. However, with increasing time this value is displaced towards a more acid pH optimum, and at the end of digestion for 16 hr. the extent of the fall in viscosity is greater at pH 4 than at pH 6.

Table 4. *Effect of various enzyme preparations on the activity of H-specific mucopolysaccharide*

Substrate (1 ml. of 1% soln.) in buffer containing 10^{-3} mM-BAL and 5 mg. of crystalline enzymes was used. Incubation, 16 hr. at 37°. Toluene was added as preservative.

Enzyme	pH	Serological activity* (μg.)
—	—	0·8
Ficin	7·6	3
Ficin	4·0	6
Papain	7·6	3
Papain	4·0	6
Lysozyme	7·6	0·8
Lysozyme	4·0	0·8
Trypsin	7·6	0·8
Trypsin	4·0	0·8
Plasmin†	7·6	0·8
Plasmin†	4·0	0·8
Pepsin	1·9	0·8
Pepsin	4·0	0·8
Chymotrypsin	7·6	0·8
Chymotrypsin	4·0	0·8
Mould protease‡	7·6	0·8
Mould protease‡	4·0	0·8

* Minimum amount required to inhibit test dose of haemagglutination.

† Activated from plasminogen (supplied by Dr M. Mackay, The Lister Institute, Elstree).

‡ Prepared from *Aspergillus oryzae* and supplied by Dr W. G. Crewther (see Crewther & Lennox, 1950).

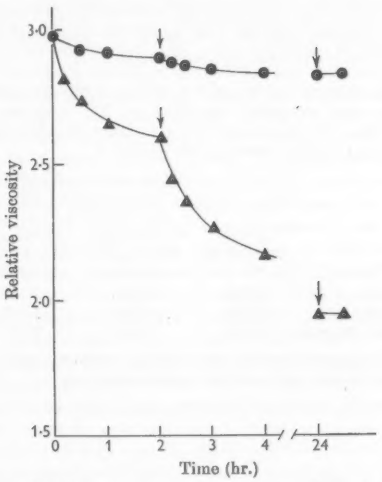

Fig. 1. Relative viscosity of an H-specific substance (2 ml. of 2%, w/v, soln.) in buffer (pH 4·0) containing 4×10^{-3} mM-BAL and different amounts of papain. At the start 0·10 mg. of enzyme was added and after 2 hr. an additional amount (0·40 mg.). The same amounts of heat-inactivated enzyme were added in the control experiment (●). Points of addition are marked with arrows. A final amount of 0·4 mg. of enzyme was added after 24 hr.

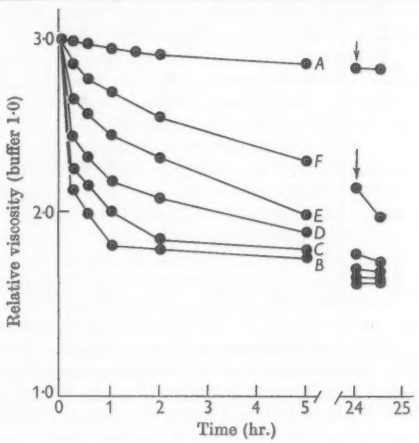

Fig. 2. Dependence of rate and extent of change in viscosity of an Le^a-specific mucopolysaccharide with enzyme:substrate ratios varying from 1:20 to 1:10 000. *A*, Control, heated enzyme:substrate ratio 1:20. *B–F*, active enzyme, ratios 1:20, 1:100, 1:1 000, 1:4 000, 1:10 000 respectively. After 24 hr. the change in viscosity brought about by the addition of 0·2 mg. of papain (enzyme:substrate ratio 1:100) is shown. Points of addition of enzyme are marked with arrows.

Table 5. *Proteolytic activity remaining after crystalline papain, pepsin and mould protease had been incubated with mucopolysaccharide*

The substrate (1 ml. of 1% soln.) in the appropriate buffer was incubated for 16 hr. at 37° with enzyme (0·5 mg.). The enzyme activity remaining was then determined by Anson's haemoglobin method.

Enzyme	Initial proteolytic activity*	Proteolytic activity* after incubation for 16 hr. Without blood-group substance	With blood-group substance
Papain	0·707	0·430	0·154
Pepsin	1·373	1·320	1·267
Mould protease	0·185	0·185	0·188

* Expressed as extinction values at 280 mμ of a 5% trichloroacetic acid-soluble digestion mixture.

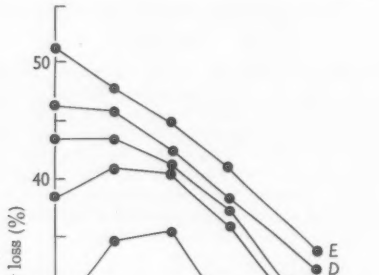

Fig. 3. pH-Dependence of the action of ficin on A substance. Each curve records the percentage viscosity loss at different pH values for a known time interval. Curves *A–E* are for 10, 30, 60 and 120 min. and for 16 hr. respectively.

H^+ *ion changes observed during the action of ficin or papain on the specific mucopolysaccharides*

Pusztai & Morgan (1958) and Morgan & Pusztai (1961) showed that the products of the action of ficin and papain on the specific mucopolysaccharides were fragments of the macromolecule, all of which retained some of their original activity and were in many respects closely similar to the starting material in chemical composition.

An attempt was made to determine the extent to which ionized groups are liberated during digestion of the mucopolysaccharides with ficin or papain. The results of an experiment with A substance and papain are given in Table 6. The acid equivalents

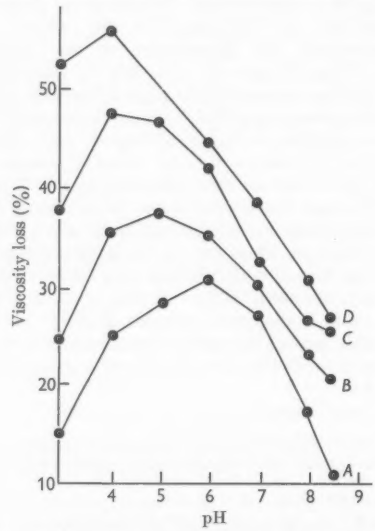

Fig. 4. pH-Dependence of the action of papain on A substance. Each curve records the percentage viscosity loss at different pH values for a known time interval. Curves *A–D* are for 10, 30 and 120 min. and for 16 hr. respectively.

Table 6. *Consumption of alkali by A-specific mucopolysaccharide during incubation with papain*

Group A substance no. 240 (3·1 g.) in 200 ml. of water containing 2 mM-BAL and 60 mg. of crystalline papain in 30 ml. of water containing 0·3 mM-BAL were used. Both solutions were made to pH 7·0, mixed together and held at 37°. NaOH (0·01 N) was added to maintain pH 7. A control mixture containing heat-inactivated (30 min. at 100°) enzyme was included.

Time (min.)	Vol. of 0·01 N-NaOH taken up (ml.) Active enzyme	Control	Acid equiv./10^5 g. Active enzyme	Control
0	0	0	0	0
2	4·65	0	1·50	0
3	5·68	0	1·83	0
5	6·62	0	2·14	0
10	7·82	0·05	2·53	<0·02
15	8·61	0·05	2·80	<0·02
30	8·90	0·05	2·90	<0·02
60	8·90	0·05	2·90	<0·02

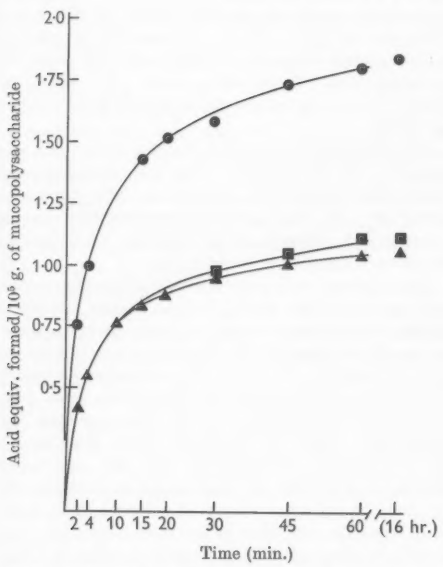

Fig. 5. Acid equivalents formed during the action of papain on three preparations of mucopolysaccharide obtained from the same cyst fluid. Curves are for materials precipitated within the ethanol concentrations 48–55% (●), 55–57% (■) and 57–65% (▲).

required for 10^5 g. of the mucopolysaccharide are about 2·9.

A similar experiment was made with three mucopolysaccharide fractions obtained from a purified Le^a mucopolysaccharide obtained from a single cyst fluid derived from a 'non-secretor'. The fractions were obtained at the final stage in purification by precipitation at 48–55, 55–57 and 57–65% (v/v) of ethanol. Each material was further fractionated two or three times at these concentrations. The analytical figures for the materials are given by Pusztai & Morgan (1961*a*). A sample (1·8 g.) of each fraction was incubated with 30 mg. of papain at pH 7·5 in a total volume of 130 ml. The formation of acid in each instance is plotted against time (Fig. 5). The addition of more crystalline papain induced no further change. The amount of H^+ ion release brought about by the heat-inactivated enzyme or by autolysis of the enzyme alone was insignificant (0·02–0·03 acid equiv./10^5 g. of substrate).

DISCUSSION

Most attempts to determine the structure of biologically active mucopolysaccharide or mucoprotein molecules have centred round the identification of those structures of the macromolecule which are associated with biological activity, and in studies on human blood-group-specific mucopolysaccharides and the virus receptors of certain mucoprotein components of human and animal secretions considerable progress has been made in identifying the individual chemical structures which are responsible for the biological activity (cf. Bettelheim-Jevons, 1958). In many instances the information was obtained through the action of carbohydrases on the specific substrates, but the nature and structure of the biologically inactive residue remaining after the release or destruction of the grouping responsible for activity received little attention. There is evidence that the structures responsible for the activity of the specific substances make up a small part only of the naturally occurring material, so that, from a chemical point of view, the overall structure of macromolecules, the specific receptors of which have been largely identified, remains almost unknown. It was earlier observed that the blood-group-specific substances were attacked by ficin and papain and certain of their physical and serological properties were modified (Lawton *et al.* 1956; Pusztai & Morgan, 1958). However, no clearly demonstrable change in the carbohydrate components of the specific substances occurred and the results indicated that the reduction of activity could therefore be achieved not only by the release of carbohydrate groupings, through the action of carbohydrases as shown earlier (Schiff, 1939; Stack & Morgan, 1949; Watkins, 1953; Howe & Kabat, 1953; and Iseki & Masaki, 1953), but also by the degradation of the mucopolysaccharide into relatively small units, presumably through the splitting of a few peptide or ester bonds only in the mucopolysaccharide macromolecule. The loss in activity induced by ficin or papain occurred irrespective of the specificity of the substance within the ABO blood-group classification, and the genetically independent Le^a blood-group character was likewise extensively destroyed by these enzymes.

Attempts to inhibit the changes induced in the specific substances by ficin or papain by the addition of simple sugars (Watkins & Morgan, 1955), or to demonstrate that these enzymes had any action on known glycosides, failed. The changes induced by ficin and papain are therefore quite different from those brought about by the enzymes of *Clostridium welchii* or *Trichomonas foetus*. The extent of serological inactivation given by different amounts of crystalline ficin or papain, and the finding that destruction occurs with an enzyme:substrate ratio as high as 1:10 000 indicates that the enzyme responsible for the changes induced is the main, SH-dependent proteolytic component of the enzyme used.

The exact nature of the chemical units involved in the linkages hydrolysed by papain and ficin, however, is not yet established. Structural and kinetic studies with crystalline papain (Kimmel & Smith, 1957) and ficin (Gutfreund, 1955; Hammond & Gutfreund, 1959) have shown that these enzymes have a broad substrate specificity. Apart from the synthetic peptide substrates studied by Dekker, Taylor & Fruton (1949) and Kimmel & Smith (1954), most of the corresponding esters and amides (Kimmel & Smith, 1954; McDonald & Balls, 1954) and certain thiol esters (Johnston, 1955, 1956) are also hydrolysed. The most sensitive substrates are the benzoyl-arginine and -lysine esters and amides. These, however, are readily hydrolysed by trypsin, whereas the specific mucopolysaccharides are not attacked by this enzyme. In view of the diversity of structures susceptible to hydrolysis by ficin or papain, it is unwise at this stage to suggest any precise structures which might be involved when these proteolytic enzymes act on the specific substances. Macromolecular structure has a considerable influence on the extent and course of action of an enzyme and linkages known to be susceptible to hydrolysis when present in small molecules are not necessarily attacked when they exist in a macromolecule.

Molecular-weight determinations on several of the specific-mucopolysaccharide preparations give values of $200–500 \times 10^3$, and this size, when considered together with the high axial ratio found (Kekwick, 1950, 1952*a*, *b*; Caspary, 1954), suggests a strongly asymmetric molecule. Such a molecular form would probably account, in part, for the high viscosity of the specific substances. As the viscosity almost certainly depends, amongst other things, on the size and shape of the macromolecule, presumably any change in these two properties brought about by enzyme action would be reflected in a change in the viscosity. The viscosity of mucopolysaccharide solutions is influenced by inorganic ions and by certain organic molecules (urea, thiourea, salicylic acid etc.), but the changes induced are usually reversible and, in most instances, the removal of the added substance by dialysis restores the original viscosity. The digestion of blood-group mucopolysaccharides with ficin or papain, however, brings about an irreversible loss in viscosity which suggests that a permanent change in the size or shape (or both) of the macromolecule has taken place. The rate of change in viscosity which occurs with different enzyme:substrate ratios, ranging from 1:10000 to 1:20, reveals the dependence of the extent of change on the concentration of enzyme in the system. Both ficin and papain show a characteristic pH optimum near 6 when viscosity measurements are made during the period of the initial reaction velocity. There is, however, a gradual displacement towards a more acid pH with increasing time of reaction. After 1 hr. the total extent of the viscosity drop is greater at pH 4 than at pH 6.

The large and irreversible change in viscosity and activity brought about by ficin and papain suggest that quite extensive changes in the molecular structure of the mucopolysaccharides occur, presumably because of the rupture of a limited number of peptide or ester bonds.

An attempt was made to determine the nature and extent of the bonds broken during the initial phase of the action of ficin or papain on the specific mucopolysaccharides. In the experiment illustrated in Fig. 5 the numbers of acid equivalents formed/10^5 g. from three mucopolysaccharides were 1·83, 1·05 and 1·10 respectively. In this instance, the molecular weight ($2·37 \times 10^5$) of the first mucopolysaccharide preparation was the only one firmly established. If this value is correct, the number of linkages broken is not less than 4 per molecule. The use of ficin in place of papain gave a similar value (3·9). The smaller uptake of alkali required by the other two fractions, which from their properties were almost certainly smaller in size than the first fraction, suggests that the number of bonds broken is probably related to the size of the substrate molecule. The materials were sialomucopolysaccharides (Pusztai & Morgan, 1961*a*), but, under the experimental conditions employed, the *N*-acetylneuraminic acid contained in the molecule was not released.

In most blood-group-specific mucopolysaccharides there are no known acidic components other than amino acids, so that one of the component units associated with the linkages split by ficin or papain must be amino acid and the point of hydrolysis must involve a carboxyl group. The other component of the linkage might be carbohydrate, but the extent to which such linkages occur and are attacked by ficin or papain is not known.

The scission of ester linkages, including glycosidic ester structures, gives CO_2^- ions, and the splitting of peptide bonds, both CO_2^- and NH_3^+ ions. If ester linkages were alone attacked, the number of bonds broken could be calculated from the alkali required to maintain the reaction at a pH range at which the CO_2H group is fully dissociated, that is at pH 5 and above, and there would be a linear relationship between the number of ester bonds split and the amount of alkali consumed. No such simple relationship holds for the hydrolysis of peptide bonds unless the dissociation of all the different amino groups liberated are completely suppressed, and this is achieved only at an alkalinity well above pH 8 (cf. Ottesen, 1958).

Unfortunately, as yet, there is no exact knowledge of the amino acids involved and hence of the pK values required to allow the determination of the number of peptide bonds broken to be made. At pH values where it is possible to neglect the ionization of the amino groups, the enzymes are so far removed from their optimum conditions that they work either poorly or not at all, and the measurement of the alkali consumed by reaction mixtures held at pH 7, as in our own experiments, therefore gives a minimum and approximate value only for the number of peptide or ester bonds (or both) broken during the initial phase of the hydrolysis.

The results, taken together, indicate that the changes in serological activity, in viscosity and in the formation of acid groups, which occur when crystalline ficin or papain act on highly purified preparations of the human blood-group-specific mucopolysaccharides, are rapid and are essentially complete in about the same time under the same conditions. The fact that these changes occur in all the mucopolysaccharides studied indicates that they most probably involve deep-seated structures which are not responsible for the primary specificity of each material but are common to all the specific substances.

SUMMARY

1. The digestion of the blood-group-specific mucopolysaccharides with activated crystalline papain or ficin at pH 4·0 or 7·6 brings about a rapid fall in viscosity and a considerable, but limited, loss in the capacity of the materials to inhibit specific haemagglutination.

2. The rate and extent of hydrolysis depend on the concentration of the enzyme and on the pH. The influence of the presence of activators and an inhibitor, *p*-chloromercuribenzoate, is considered.

3. Trypsin, chymotrypsin, plasmin, pepsin and a mould protease appear to have no action on the group-specific mucopolysaccharides.

4. The results suggest that there are a limited number of peptide bonds broken. The minimum number is probably about four in the initial phase of the reaction for a mucopolysaccharide of about $2{\cdot}50 \times 10^5$ molecular weight.

5. The changes in viscosity and serological activity and the liberation of titratable groups occur simultaneously and early in the hydrolysis and take place irrespective of the serological specificity of the mucopolysaccharide. These findings suggest that the kind of linkages broken are common to all the group-specific substances tested and therefore involve structures not primarily responsible for serological specificity.

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Studies in Immunochemistry

21. THE PRODUCTS OF THE DEGRADATION OF BLOOD-GROUP-SPECIFIC SUBSTANCES BY FICIN AND PAPAIN

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In earlier work (cf. Morgan, 1954–55; Kabat, 1956; Morgan, 1960) it was shown that the blood-group-specific serological properties of human secretions and body fluids are associated with macromolecular entities which contain carbohydrate and amino acids firmly bound together, and that the part of these complex molecules which is primarily responsible for the characteristic serological behaviour is carbohydrate in nature. There is some evidence, however, that not only is the nature, the mode of linkage and the sequence of the sugar units of importance in the qualitative and quantitative behaviour of the specific substances with their antibodies, but that the orientation of these structures and the overall conformation of the macromolecule play an important part and are probably maintained by the amino acid-containing moiety of the specific substance.

In order to study the part played by the amino acids in mucopolysaccharide structure, the action of the proteolytic enzymes, ficin and papain, on the specific substances was examined (Pusztai & Morgan, 1958, 1961*c*), and it was found that these enzymes brought about a rapid reduction in the specific serological activity to a value equivalent to a few per cent only of the original figure, when the activity was measured by the haemagglutination-inhibition test. Repeated incubation with fresh enzyme, however, failed to destroy completely the serological activity of the specific substances. These changes occurred irrespective of the specificity of the substances, and the different materials examined were all broken down into smaller, non-diffusible fragments which in each instance had a chemical composition closely similar to that of the substance from which it was obtained.

A more extensive series of experiments has now been made and the results obtained with several highly purified specific substances and crystalline ficin and papain are now given.

EXPERIMENTAL

Group-specific substances. These materials were isolated from human ovarian-cyst fluids and purified as described by Morgan & King (1943), Gibbons, Morgan & Gibbons (1955) and Pusztai & Morgan (1961*b*). Except where stated otherwise, all materials were insoluble in 90% (w/w) phenol, soluble in water, insoluble in saturated $(NH_4)_2SO_4$ at 60° and precipitated from solution (1% in water) within a narrow range of ethanol concentration. The substances were electrophoretically homogeneous at pH 8 in phosphate buffer, *I* 0·2.

Physical measurements. Ultracentrifugal, electrophoretic and diffusion measurements were made as described by Kekwick (1950, 1952*a*, *b*) and Caspary (1954). Intrinsic viscosities were determined from relative-viscosity measurements made at 20° and pH 8 in phosphate buffer (*I* 0·2) containing 0·15M-NaCl (Pusztai & Morgan, 1961*a*, *b*). Partial specific volumes were determined pyknometrically.

Chemical analysis. The following methods were used. Nitrogen: Kjeldahl determination (Markham, 1942). Fucose; Gibbons (1955). Amino sugars: Rondle & Morgan (1955). Reducing sugars: Nelson (1944). The last two determinations were made after the material had been hydrolysed in sealed ampoules with 0·5N-HCl at 100° for 18 hr. and the results are expressed throughout as glucosamine base or glucose respectively.

Quantitative amino acid composition was determined as described by Moore, Spackman & Stein (1958) with a column (150 cm.) of Aminex, MS Blend Q-150, particle size 31–45 μ, for acidic and neutral amino acids, and Aminex, MS Blend Q-50, particle size 25–31 μ, for the short (15 cm.) column for basic amino acids.

Serological activity. The serological activity was determined by the capacity of the materials to inhibit the agglutination of a 1% suspension of erythrocytes by the appropriate A, B or Le^a human antibody (Gibbons *et al.* 1955). H activity was measured with rabbit immune H antibody (Annison & Morgan, 1952; Watkins & Morgan, 1955).

Enzymes. Crystalline (×2 or ×3) papain and ficin, as described earlier, were used (Pusztai & Morgan, 1961*c*). The enzymes were activated with 2,3-dimercaptopropanol (BAL) immediately before use.

Enzymic hydrolysis. A known weight of the specific substance was dissolved in 0·1M-citrate–phosphate buffer, pH 4·0, containing BAL (Pusztai & Morgan, 1961*c*) to give a 1 or 2% (w/v) solution and incubated with enzyme (1, 2 or 5%, w/w, of substrate). Toluene was added as preservative. In some experiments the substrates were incubated two or more times with enzyme. Where this occurred the material was dialysed and the pH adjusted before the addition of fresh enzyme.

Isolation of the hydrolysis products. After incubation with enzyme the material was dialysed, saturated with $(NH_4)_2SO_4$ and heated to 60° for 5–10 min. This treatment

denatured and precipitated the enzyme protein and, where the specific substance was originally insoluble under these conditions, removed unchanged substrate. The specific substances which were originally insoluble in saturated $(NH_4)_2SO_4$ at 60° became completely soluble in this reagent after thorough incubation with papain or ficin. The $(NH_4)_2SO_4$ was removed by dialysis at 1–3°, the hydrolysis products were recovered by drying from the frozen state, dissolved to give a solution (1 or 2%, w/v) in water and fractionated by the addition of ethanol containing 1% of potassium acetate. The main fractions obtained were frequently refractionated within the same limits of ethanol concentration. The materials finally obtained were dissolved in water, thoroughly dialysed and dried from the frozen state.

RESULTS

A preparation of group A substance obtained from cyst fluid no. 346 was incubated with ficin at pH 4·0, and after thorough dialysis again treated with ficin under the same conditions. The enzyme and any unchanged substrate were removed as described and the products separated into four fractions by the addition of ethanol (Table 1). The original substrate was completely precipitated from solution between 50% and 55% of ethanol, whereas after treatment with ficin slightly more than half of the substance only could be recovered between these concentrations of ethanol. The remaining material precipitated on the addition of higher concentrations of ethanol and about 20% of the starting material was recovered between 60% and 75% of ethanol. Only about 2% of the material was soluble above 75% of ethanol. Nevertheless, in spite of the changed solubility, the chemical composition of the materials, expressed in terms of fucose and total amino sugar and reducing sugar, remained largely unchanged. The nitrogen values of the recovered materials, however, were less than that of the original substance by a significant amount.

The products of degradation all had a lower relative viscosity than did the original substance, and the more soluble the fractions were in ethanol, the lower was their viscosity. Similarly, all the fractions obtained were less active than the original material, and the first and largest fraction and the last and most soluble fraction possessed no more than about 3% or less than 0·1% respectively of the activity of the starting material.

The material (248 mg.) insoluble in saturated ammonium sulphate at 60° after incubation with enzyme contained the denatured enzyme and some unchanged specific substance. It was recovered, incubated again with ficin and the products were added to the main hydrolysis products and included in the ethanol fractionation which finally gave the materials described in Table 1. The overall yield of recovered substance was rather more than 80% of the original substrate.

A part (550 mg.) of the material (Table 1) precipitating between 50% and 55% (v/v) of ethanol was dissolved in citrate–phosphate buffer and again incubated with ficin for 24 hr. The solution was then saturated with ammonium sulphate, heated to 60° and the soluble material recovered after dialysis and drying from the frozen state and fractionated from solution in water by ethanol. The substrate is seen (Table 2) to be degraded in much the same manner as was the original specific substance, although the formation of material (13%) soluble in 60% ethanol was reduced in amount compared with that (20%) resulting after the first treatment with ficin. The fractions are again found to be closely similar in chemical composition to the original substance.

A portion (350 mg.) of the material which was precipitated between 50% and 55% (v/v) of ethanol (Table 2), after incubation three times with ficin, was again dissolved in buffer and incubated for 16 hr. with 17 mg. of papain. The

Table 1. *Analytical values of materials produced from blood-group A substance no.* 346 *after digestion with ficin*

The A substance (1·2 g.) was incubated with ficin (21 mg.) in 0·1 M-citrate–phosphate buffer, pH 4, for 48 hr. Products were recovered and treated again with ficin (21 mg.). The digest was fractionated with ethanol.

		Fractions precipitated			
Concn. of ethanol (%, v/v)	Original substance	50–55	55–60	60–75	Above 75
Material (mg.)	1200	664	98	212	23
Nitrogen (%)	5·3	4·6	4·8	4·8	5·2
Fucose (%)	19·4	21·3	21·1	20·1	16·0
Hexosamine (%)	27·7	29·4	28·5	26·2	22·0
Reducing sugar (%)	48·5	54·3	54·9	52·2	49·6
Viscosity (rel. *c*, 1% in water)	3·12	1·67	1·24	1·12	—
A activity* (μg.)	0·025	0·8	12	50	50

* End point of inhibition against natural human anti-A serum.

Table 2. *Analytical figures of material precipitated from A substance after further digestion with ficin*

Material (550 mg.; 50–55% ethanol precipitate, Table 1) was incubated with ficin (10 mg.) in 0·1 M-citrate buffer, pH 4, for 24 hr. and fractionated with ethanol.

Concn. of ethanol (%, v/v)	Material before treatment	Fractions precipitated 50–55	55–60	60–75
Material (mg.)	550	390	35	75
Nitrogen (%)	4·6	4·6	4·7	4·8
Fucose (%)	21·3	22·3	20·5	22·2
Hexosamine (%)	29·4	28·9	25·6	28·3
Reducing sugar (%)	54·3	53·3	50·9	53·8
Viscosity (rel. *c*, 1% in water)	1·67	1·36	1·18	1·16
A activity* (μg.)	0·8	0·8	12	25

* See footnote to Table 1.

material (300 mg.) precipitated by ethanol (50–55%, w/v) made up nearly 90% of the starting material and was essentially unchanged in chemical composition and activity (Table 3). The material is apparently resistant to further degradation with ficin or papain at pH 4.

The A substance no. 401, insoluble in 90% phenol, soluble in water and insoluble in saturated ammonium sulphate at 60°, was fractionated from ethanol, and the main fraction, collected between 45% and 50% (v/v) of ethanol concentration, was precipitated three times within this ethanol range from solution in water containing 1% of potassium acetate. The material (2 g.) recovered was thoroughly dialysed, dried from the frozen state and incubated with three successive portions (30 mg.) of ficin, dialysed and recovered. The digestion products were then treated with saturated ammonium sulphate at 60° and separated into material insoluble (0·21 g.) and soluble (1·68 g.) under these conditions. The insoluble material was incubated with 5 mg. of ficin and finally yielded an additional 130 mg. of substance soluble in saturated ammonium sulphate. The total yield of mucopolysaccharide soluble in ammonium sulphate from the original material (2 g.) was 1·8 g. This substance (1·72 g.) was fractionated with ethanol and gave no precipitate up to 48% (v/v) of ethanol, 1·50 g. between 48% and 53% (v/v) of ethanol, no precipitate between 53% and 58% (v/v) of ethanol and 0·19 g. soluble in 58% (v/v) of ethanol. Both materials were incubated again with ficin and recovered after fractionation with ethanol. The overall yield was 1·30 g., precipitating between 49% and 53% (v/v) of ethanol, and 0·26 g. between 58% and 75% (v/v) of ethanol. An attempt to refractionate the latter material gave 100 mg. of substance between 58% and 62% of ethanol, 130 mg. between 62% and 66% of ethanol and 13 mg. between 66% and 70% of ethanol. The first two fractions were combined, as they were considered to be parts of one main fraction. The analytical values of those materials finally collected between 49% and 53% and between 58% and 66% of ethanol are given in Table 4. The original mucopolysaccharide material is seen to be divided into two fragments, the major material being precipitated at a lower ethanol concentration than that required to precipitate the second and minor component.

Table 3. *Analytical figures of the main product obtained from A substance after digestion with ficin and papain*

Material (350 mg.; 50–55% ethanol precipitate, Table 2) was incubated with papain (17 mg.) in 0·1 M-citrate-phosphate buffer, pH 4, at 37° for 16 hr.

	Material before (*a*) and after (*b*) incubation with papain and precipitation between 50% and 55% of ethanol (*a*)	(*b*)
Material (mg.)	350	300
Nitrogen (%)	4·6	4·6
Fucose (%)	22·3	21·8
Hexosamine (%)	28·9	29·5
Reducing sugar (%)	53·3	54·5
Viscosity (rel. *c*, 1% in water)	1·36	1·42
A activity* (μg.)	0·8	0·8

* See footnote to Table 1.

The behaviour of two mucopolysaccharide materials isolated from an ovarian-cyst fluid obtained from a person of blood group A_2B (cyst no. 186) was also studied. In this instance, the specific substances investigated included materials which were (*a*) insoluble and (*b*) soluble in saturated ammonium sulphate at 60° (cf. Pusztai & Morgan, 1961*b*). The materials were each separately incubated at pH 4 with papain and the products were separated and recovered, as described above. The composition and properties of the materials (Table 5) show that the changes occurring in both

materials are essentially similar to those found for the group A substances already described (Tables 1–4).

The group A_1B substance from cyst fluid no. 117 was likewise incubated at pH 4·0 with ficin, dialysed and treated with a second similar quantity of enzyme before the products were recovered.

Table 4. *Analytical values of A substance no.* 401 *and the two main decomposition products produced by ficin*

The A substance (2 g.) was incubated repeatedly with ficin and the products were fractionated with ethanol.

Concn. of ethanol (%, v/v) ...	Original substance	Fractions precipitated 49–53	58–66
Material (mg.)	2000	1300	230
Nitrogen (%)	4·9	4·4	4·2
Fucose (%)	22·5	22·1	19·1
Hexosamine (%)	28·5	28·4	24·2
Reducing sugar (%)	51	52	45

Details of the materials obtained are given in Table 6, and show again that the changes induced are similar to those described above.

The results obtained when H-specific substance no. 277 was incubated with three successive quantities of papain at pH 4·0 and the products were separated and examined are given in Table 7. It is again evident that the original material was broken down into smaller units and that the composition of each of the products remained closely similar to that of the original material.

The incubation of an Le^a-specific mucopolysaccharide with papain has already been described (Pusztai & Morgan, 1961*a*), and analytical details of the products are given in Table 9 in that paper. The changes induced by the enzyme are similar to those described above for the other specific substances.

Behaviour of mucopolysaccharide preparations of different size on incubation with papain

The influence of the size of the mucopolysaccharide molecule on the extent of molecular degradation achieved was studied in the following

Table 5. *Analytical values of materials recovered after incubating a blood-group A_2B substance with papain*

Materials (*a*) insoluble and (*b*) soluble in saturated ammonium sulphate at 60° were each digested with papain (10 mg.) and subsequently fractionated with ethanol.

Concn. of ethanol (%, v/v)	Original substance	(*a*) 50–55	55–60	60–75	Above 75	Original substance	(*b*) 50–55	55–60	60–75	Above 75
Material (mg.)	530	338	41	62	13	530	300	60	130	7
Nitrogen (%)	5·1	4·6	4·6	4·6	4·2	3·9	3·6	3·5	3·5	3·7
Fucose (%)	20·1	21·5	19·5	19·5	—	22·2	24·6	23·6	24·2	—
Hexosamine (%)	26·0	27·4	25·8	27·4	—	25·5	29·1	29·4	29·0	—
Reducing sugar (%)	49·6	50·0	54·0	52·7	—	57·8	58·0	54·0	57·3	—
Viscosity (rel. *c*, 1% in water)	2·60	1·80	1·30	1·16	—	1·50	1·45	1·24	1·08	—
$[\alpha]_D$ (*c*, 1% in water)	−22°	−9°	−11°	−17°	—	−43°	−36°	−41°	−48°	—
A activity* (μg.)	1·0	125	250	250	250	16	>1000	>1000	>1000	>1000
H activity† (μg.)	1·6	6·0	12·5	25	25	3	25	100	100	100

* See footnote to Table 1. † End point of inhibition against rabbit immune anti-H serum.

Table 6. *Analytical values of materials produced from an A_1B-specific substance after digestion with ficin*

Material was incubated twice with ficin (2%) and fractionated with ethanol.

Concn. of ethanol (%, v/v)	Original material	Fractions precipitated 47–50	50–55	55–60	60–75	Above 75
Material (mg.)	640	19	233	100	140	16
Nitrogen (%)	5·4	4·0	3·8	4·5	4·9	5·3
Fucose (%)	18·2	17·7	19·9	18·0	15·0	12·6
Hexosamine (%)	26·6	27·0	27·8	29·4	22·3	18·7
Reducing sugar (%)	49·5	48·1	53·1	53·2	42·3	43·2
Viscosity (rel. *c*, 1% in water)	4·18	—	2·33	1·42	1·14	—
A activity* (μg.)	0·03	0·25	0·25	1·0	15	30
B activity† (μg.)	2·0	4·0	30	250	500	500

* See footnote to Table 1. † End point of inhibition against natural human anti-B serum.

Table 7. *Analytical values of materials recovered after H substance no. 277 had been repeatedly incubated with papain*

Substrate was incubated in 0·1 M-citrate–phosphate buffer, pH 4, with 2% of papain for 16 hr. at 37°. The material was recovered and digested twice more with papain under the same conditions.

		Fractions precipitated				
Concn. of ethanol (%, v/v)	Original substance	47–50	50–55	55–60	60–75	Above 75
Material (mg.)	1500	530	240	100	110	20
Nitrogen (%)	5·3	5·0	5·3	5·0	5·1	5·0
Fucose (%)	18·5	19·1	17·0	15·4	15·6	—
Hexosamine (%)	28·0	28·1	28·2	25·9	25·3	—
Reducing sugar (%)	50·3	53·5	50·0	49·5	50·3	—
Viscosity (rel. *c*, 1% in water)	5·08	1·90	1·49	1·30	1·14	—
$[\alpha]_D$ (*c*, 1% in water)	−39°	−18°	−13°	−16°	−21°	—
$S_{20,w}$ values at concn. (g./100 ml.):						
1·0	4·09	3·72	3·74	3·05	2·05	—
0·5	5·61	4·84	—	—	—	—
0·38	5·63	—	—	—	—	—
0·25	6·77	6·19	—	—	—	—
H activity* (μg.)	0·05	0·4	0·4	0·8	3·0	6·0

* End point of inhibition against rabbit immune anti-H serum.

Table 8. *Digestion of mucopolysaccharides of different size with papain*

Materials were obtained from mucopolysaccharide preparation (no. 350) by fractionation with ethanol. Each material was incubated separately with papain in citrate–phosphate buffer (pH 4) for 24 hr. Each digest was then fractionated within the same range of ethanol concentrations.

	Ethanol (%, v/v) fractionation limits of mucopolysaccharide materials before incubation with papain					
Limits (%, v/v) of ethanol fractionation after incubation with papain	48–55		55–57		57–65	
	Yield after incubation with papain and fractionation with ethanol					
	g.	%	g.	%	g.	%
48–55	1·087	64·0	—	—	—	—
55–58	0·133	7·8	1·225	73·0	0·286	16·8
59–65	0·432	25·4	0·400	23·0	1·300	76·4
68–75	0·040	2·2	0·064	3·4	0·106	6·2
Soluble in 75	0·011	0·6	0·011	0·6	0·012	0·6

experiment. A large preparation of Le^a-specific substance which was obtained from cyst no. 350 (Pusztai & Morgan, 1961*a*, *c*) and was insoluble in 90% phenol and in saturated ammonium sulphate at 60° was separated into three fractions by precipitation from water with ethanol concentrations of 48–55, 55–57 and 57–65% (v/v) respectively. Portions (1·8 g.) of each of these materials were separately incubated with papain, dialysed and recovered. Each material was dissolved in water (1%, v/v) and fractionated within the same arbitrarily chosen range of ethanol concentrations, and the yield of each fraction was recorded (Table 8).

Molecular size of the specific substances before and after incubation with ficin or papain

The specific mucopolysaccharides after incubation with ficin or papain possess a reduced viscosity, an increased solubility in water–ethanol mixtures and become completely soluble in saturated ammonium sulphate at 60°, but nevertheless show no gross change in chemical composition. These changes suggest that the original materials are broken down to mucopolysaccharide entities of smaller molecular size. The analytical values obtained for H substance no. 277 and for the materials obtained from it after incubation with papain are given in Table 7. To obtain some evidence for the molecular size of the materials obtained, the sedimentation coefficient of each fraction was examined at 1% (w/v) concentration. In some instances values at lower concentrations were also obtained (Table 7). The preparations showed a good degree of homogeneity, but were somewhat polydisperse. The last fraction obtained, which was soluble in 75% (v/v) of ethanol, gave unsatisfactory results

because of its rapid diffusion in the ultracentrifuge cell. The sedimentation coefficients obtained for the other materials are given in Table 7, and the values obtained support the view that all the fractions recovered after incubation with papain are smaller than the original H substance. The intrinsic viscosity (0·62) of the H substance and of its largest fragment (0·445) obtained after incubation with papain were determined by a linear extrapolation to zero concentration of $\eta_{spec.}/c$ values plotted against concentration. The sedimentation coefficients obtained at the lowest dilution examined (0·25%), together with the $\eta_{spec.}/c$ values at the same concentration, the partial specific volume (0·63) and the densities of the solutions were substituted in the formula:

$$M = \frac{4690 \times (S_{20,w})^{\frac{1}{2}} \times [\eta]^{\frac{1}{2}}}{(1-\bar{V}\delta)^{\frac{1}{2}}}$$

(cf. Schachman, 1957), to obtain molecular weights. The values obtained were 340 000 and 271 000 respectively for the original H substance and the largest of the decomposition products obtained from it after incubation with papain.

In a similar experiment H substance (no. 277) was incubated with ficin at pH 4 and the molecular weights of the original material and of the main decomposition product were determined. The examination was made by Mr E. Caspary, who determined the diffusion coefficients and used these in place of the intrinsic-viscosity values to calculate the molecular weight. The results are given in Table 9. The diffusion coefficients could not be extrapolated to zero concentration in a linear manner and the molecular weights are therefore calculated from the Svedberg formula

$$\frac{RTS^0_{20}}{D^0_{20}(1-\bar{V}\rho_{20})}$$

with diffusion coefficients obtained for the lowest concentration and the appropriate sedimentation constant. The molecular weights obtained are 332 000 and 265 000 for the original H substance and the main decomposition product. The frictional values are 3·23 and 2·85 respectively. The results of these two experiments, in which either papain or ficin was used to bring about the degradation, showed that the largest of the products had a molecular weight which was smaller than that of the original specific substance and that the molecular size of the largest fragment was closely similar whichever enzyme was used.

A similar examination was made with a group A substance no. 346 (Table 1) and its main ficin and papain decomposition product (Table 3, *b*). In this instance the extent of the degradation, as determined from the measurements given in Table 10, appears to be greater than was found for

Table 9. *Physical properties of H-specific mucopolysaccharide and the main degradation product formed on incubation with ficin*

(1) and (2) Sedimentation coefficients. (3) Diffusion coefficients. (4) Frictional ratios. (5) Molecular weights of the (*a*) original and (*b*) the ficin-degraded H substance obtained from cyst fluid no. 277.

Concn. (mg./ml.)	(*a*)	(*b*)
(1) Sedimentation coefficients at pH 8·0, *I* 0·35		
10·0	4·89	4·64
7·5	5·49	5·14
5·0	6·25	6·05
2·5	6·87	6·50
Infinite dilution	7·51	7·30
(2) Sedimentation coefficients at pH 4·0, *I* 0·35		
10·0	4·81	4·81
5·0	6·24	6·01
2·5	7·54	6·75
(3) Diffusion coefficients at pH 8·0, *I* 0·35		
4·6	1·22	—
3·3	1·32	—
1·5	1·51	—
4·1	—	1·52
3·1	—	1·61
1·5	—	1·84
(4) Frictional ratio	3·23	2·85
(5) Molecular weights	332 000	265 000

Table 10. *Physical properties of A-specific mucopolysaccharide and the main degradation product formed on incubation with papain*

(1) Sedimentation coefficients. (2) $\eta_{spec.}/c$. (3) Partial specific volumes. (4) Calculated molecular weights of (*a*) original and (*b*) ficin-degraded A substance obtained from cyst fluid no. 346.

	(*a*)	(*b*)
(1) Sedimentation coefficients at pH 8·0, *I* 0·35		
Concn. (in refractive increments)		
0330	3·65	—
0200	4·70	4·20
0150	5·20	4·57
0100	—	5·06
Infinite dilution	6·71	5·93
(2) $\eta_{spec.}/c$ values at pH 8·0, *I* 0·35		
Concn. (mg./ml.)		
10·0	1·240	0·296
5·0	1·018	0·254
2·5	0·896	0·228
1·25	0·840	0·208
0·625	0·800	—
Infinite dilution	0·777	0·198
(3) Partial specific volumes at pH 8·0, *I* 0·35		
Concn. (mg./ml.)		
10·0	0·69	0·69
(4) Molecular weights	416 000	173 000

H substance no. 277, the molecular weight of the main decomposition product (173 000) being only about 40% of the value (416 000) obtained for the original substance. The molecular weight of the decomposition product precipitated between 60 and 75% of ethanol was examined in the Spinco analytical ultracentrifuge and from its behaviour was considered to be close to 25 000.

Amino acid composition of the fragments obtained from the specific mucopolysaccharides

Group A substance no. 346 was repeatedly treated with ficin and finally with papain, and separated into two main fractions: a material precipitating between 50% and 55% (v/v) of ethanol and a smaller component separating at a higher (60–75%) ethanol concentration (Tables 1–3). The original substance and the two decomposition products were separately hydrolysed in 6N-hydrochloric acid at 116–120° for 24 hr. and each was subjected to quantitative amino acid analysis (Moore *et al.* 1958). The results (Table 11), as expected from the lower nitrogen content of the two decomposition products, show that these each contain less total amino acid on a weight basis than does the original material and show, moreover, significant differences in amino acid distribution. Thus in the original A substance, the proportion of the amino acids in the group serine, threonine and proline, is 45%, whereas acidic amino acids and basic amino acids amount to 14% and 19% respectively. The corresponding groups in the main decomposition product are 53%, 12% and 14%, and the distribution in the smallest fragment, 35%, 20% and 22%. The amino acid composition of A substance no. 401 and its two fragments is given in Table 11. The proportion of the total amino acids in the serine, threonine and proline group is 46%; the acidic amino acid and the basic amino acids account for 11% and 20% respectively. The first and largest breakdown product contains 61% of the amino acids of the former group and 6% and 11% of acidic and basic amino acids. The smallest of the decomposition products contains 47% of the total amino acid in the serine, threonine and proline group, whereas acidic and basic amino acids represent 12% and 17% respectively of the total amino acids.

Group A substance obtained from cyst fluid no. 332 was separated into material (*a*) insoluble and (*b*) soluble in saturated ammonium sulphate at 60° (Pusztai & Morgan, 1961*b*; Table 1). Each material was incubated with ficin and fractionated into substances precipitating at different ethanol concentrations, as described throughout this paper. The original substances (*a*) and (*b*) and the main decomposition product from each, which precipitated between 50% and 55% (w/v) of ethanol, were subjected to quantitative amino acid analysis. The main decomposition product examined ac-

Table 11. *Amino acid analysis of A substances no.* 346 *and no.* 401 *and each of their two main decomposition products obtained after repeated incubation with ficin at* pH 4·0

Results are expressed as g. of amino acid/100 g. of blood-group substance.

	No. 346			No. 401		
	Original substance*	Decomposition products*		Original substance†	Decomposition products*	
Concn. of ethanol (%, v/v) ...	50–55	50–55	60–75	45–50	48–53	58–66
Amino acid						
Aspartic acid	0·95	0·62	0·94	0·71	0·25	0·49
Threonine	3·38	2·98	2·03	3·57	3·33	2·07
Serine	2·07	1·66	1·14	2·33	1·95	0·87
Glutamic acid	1·33	0·93	1·54	0·94	0·36	0·58
Proline	1·64	2·01	1·15	1·64	1·45	1·74
Glycine	0·50	0·45	0·37	0·46	0·33	0·42
Alanine	0·92	0·92	1·06	0·89	0·78	0·92
Valine	0·66	0·42	0·42	0·73	0·48	0·65
Methionine	0·05	<0·05	0·09	<0·05	<0·05	<0·05
Leucine	0·56	0·34	0·38	0·58	0·24	0·26
Isoleucine	0·40	0·19	0·20	0·46	0·32	0·18
Tyrosine	0·20	0·10	<0·10	0·12	<0·05	<0·05
Phenylalanine	0·29	0·18	0·16	0·33	0·09	0·10
Lysine	0·86	0·51	0·50	1·11	0·24	0·29
Histidine	0·82	0·48	0·67	1·01	0·48	0·61
Arginine	0·82	0·52	1·31	0·72	0·30	0·64
Unknown basic‡	0·43	0·30	0·28	0·38	0·25	0·16
	15·88	12·66	12·34	16·03	10·95	10·08

* Values are not corrected for losses on hydrolysis.
† Corrected and extrapolated values determined from 24 and 48 hr. hydrolysis.
‡ Expressed as leucine.

Table 12. *Amino acid composition of the largest degradation product obtained from A substance no. 332 after incubation with ficin*

The group A mucopolysaccharide (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60° was incubated with papain at pH 4 in citrate–phosphate buffer for 24 hr. The products were fractionated with ethanol and material was precipitated between 50% and 55% of ethanol from (*a*) and (*b*) and analysed for amino acid composition. Results are expressed as g. of amino acid/100 g. of substance.

	(*a*)		(*b*)	
Amino acid	Without ficin	With ficin	Without ficin	With ficin
Aspartic acid	0·31	0·23	0·46	0·39
Threonine	4·05	3·46	2·90	2·83
Serine	2·16	1·98	1·57	1·58
Glutamic acid	0·72	0·45	0·82	0·71
Proline	2·04	1·60	1·62	1·58
Glycine	0·43	0·35	0·37	0·35
Alanine	0·92	0·73	0·83	0·72
Valine	0·59	0·44	0·41	0·40
Methionine	0·11	0·13	0·12	0·08
Isoleucine	0·41	0·29	0·31	0·31
Leucine	0·35	0·24	0·35	0·35
Tyrosine	0·15	0·07	0·08	0·08
Lysine	0·29	0·14	0·26	0·22
Phenylalanine	0·35	0·27	0·27	0·27
Histidine	0·70	0·52	0·78	0·81
Arginine	0·55	0·32	0·44	0·35

counted, in each instance, for about 60% of the original material. The results (Table 12) show again that, as expected from the lower nitrogen content found for the decomposition products, these materials contain less amino acid than does the original substance. The results also indicate that the amount of certain amino acids in the main decomposition products is considerably reduced compared with that in the parent mucopolysaccharide. The changes are most pronounced in the material originally insoluble in hot ammonium sulphate where it is seen that this material contains 0·5 g. of threonine, 0·2 g. of serine and 0·27 g. of glutamic acid (per 100 g. of total substance) more than is contained in the decomposition product. Some of the missing amino acids could be in the materials precipitating at higher ethanol concentration and not subjected to amino acid analysis, but presumably not all, as their content of amino acid is also lower than that of the original mucopolysaccharide. These analyses were made early in the investigation and figures for the content of the unidentified basic substance (Table 11) were not obtained.

DISCUSSION

The determination of the ultimate structure of biologically important macromolecules is more and more becoming a study of the products of the stepwise breakdown of these substances whether they are obtained by acid, alkaline or enzymic hydrolysis. In the field of mucopolysaccharide structure, partial acid hydrolysis or the action of carbohydrases have been extensively employed to this end and especially to determine the precise nature of the carbohydrate units responsible for the primary specificity of the naturally occurring blood-group-specific macromolecules (cf. Morgan, 1960). The nature and function of the amino acid-containing part of the mucopolysaccharides has also been examined (Pusztai & Morgan, 1958) by studying the action of ficin and papain on the specific substances.

The carbohydrases split many and different glycosidic linkages in the macromolecules, mainly in the terminal parts of the sugar chains, and released sugar units. Ficin and papain, on the other hand, hydrolysed only a small and limited number of linkages, most probably those which are intimately associated with the internal structure of the specific substance, and therefore gave rise to only a small amount of diffusible material.

The mucopolysaccharides after thorough incubation with ficin or papain at pH 4 showed marked changes in certain of their properties and always possessed a lower viscosity and a much reduced serological activity (Pusztai & Morgan, 1958, 1961*c*). In order to study these and other changes in greater detail, preparations of specific mucopolysaccharides which were free from gross heterogeneity (Pusztai & Morgan, 1961*b*) were exhaustively treated with ficin or papain or both and the products recovered and examined.

In most instances the mucopolysaccharides studied were insoluble in saturated ammonium sulphate at 60°. After incubation with ficin or papain they became soluble in ammonium sulphate and had a lower content of nitrogen, were less active serologically and were much reduced in viscosity. It was shown (Pusztai & Morgan, 1961*b*) that, besides a mucopolysaccharide that was insoluble in ammonium sulphate at 60°, there existed in many cyst fluids a mucopolysaccharide material which was soluble, and that such substances also contained less nitrogen than the substances which were insoluble in ammonium sulphate at 60°, and were obtained from the same cyst fluid. These materials were less active and had a lower viscosity than did the materials insoluble in hot ammonium sulphate. The soluble materials on treatment with ficin or papain behaved as did the corresponding mucopolysaccharide which was insoluble in ammonium sulphate, and the products of digestion likewise were less active and had a lower viscosity than did the starting material. It appears therefore that not only the structures responsible for making a material insoluble in

saturated ammonium sulphate are changed by ficin or papain, but that similar or other structural features which are associated with the entities soluble in ammonium sulphate are also modified. It is possible therefore that two types of structure are involved in the changes induced.

The specific substance, which exists in a form which is either insoluble or soluble in saturated ammonium sulphate at 60° (Pusztai & Morgan, 1961*b*), is precipitated by ethanol from solution in water within quite narrow limits of ethanol concentration. After thorough and repeated incubation with ficin or papain, the products obtained from either material precipitate over a much wider ethanol range and, in order to examine the products, they were divided by ethanol concentration into four or five arbitrarily chosen fractions and each material was recovered, analysed and the relative viscosity and the serological activity were determined. The yields of the different fractions recovered showed that two main materials were obtained. One, which usually accounted for about two-thirds of the original substance, was precipitated at a somewhat higher ethanol concentration than was needed to precipitate the original substance. The second fraction precipitated at a considerably higher concentration of ethanol, usually between 60% and 75% ethanol. This material makes up about 20% of the starting substance. Frequently material was precipitated at ethanol concentrations in between those required to obtain the two major fractions, but the amount was usually small and, together with any material remaining soluble in 75% of ethanol, seldom amounted to more than 20% or so of the total substance involved. Refractionation of the material collected over the arbitrarily selected range of 60% and 75% of ethanol gave a material which precipitated between quite narrow limits, usually 59% and 65%.

It seems reasonable to believe therefore that as a result of thorough proteolytic decomposition two kinds of mucopolysaccharide fragments are formed and that the occurrence of any additional components can be explained as follows. First, the fractionation with ethanol is not a precise procedure and some carry-over from fraction to fraction is certain to occur. This error can be largely eliminated by refractionating the materials over the same ranges of ethanol concentration. Secondly, it has already been stressed (Pusztai & Morgan, 1961*a*, *b*) that the blood-group mucopolysaccharides obtained from ovarian-cyst fluids give preparations which contain chemically similar or identical molecules but of very different size. With improved fractionation procedures, such as that employing saturated ammonium sulphate at 60° and high-speed centrifuging, the polydispersity of a starting material can be reduced to a level where a preparation has 80–90% of its molecules closely similar in size (Pusztai & Morgan, 1961*a*, Fig. 2; Pusztai & Morgan, 1961*b*, Fig. 3); the remaining material, however, has a very different molecular size and contains both larger and smaller units. It might be expected that the enzymic degradation products of these different-sized mucopolysaccharides are of different size and will, in consequence, show different precipitation behaviour with ethanol. This suggestion is supported by the data obtained (Table 8) from an experiment in which three mucopolysaccharide preparations, each obtained from the same cyst fluid, but judged from their viscosity values to be of different average molecular size, were examined separately after incubation with papain. The amount of material (6·2%) precipitated within the ethanol range 68–75%, which was obtained from an original fraction which itself was precipitated within the range 57–65% (Table 8), can be compared with the amount of material (2·2%) precipitated over the same ethanol range (68–75%) but obtained from a mucopolysaccharide, which was precipitated from the original mucopolysaccharide at a lower ethanol concentration (48–55%) (Table 8). It appears that the smaller the molecular size of the starting material, the larger is the yield obtained of the smaller and most soluble (in ethanol) material. Similar conclusions can be drawn from the results given in Table 5, where after treatment with papain the yield of the 60–75% ethanol fraction (62 mg.) obtained from material insoluble in saturated ammonium sulphate at 60° is seen to be less than half that isolated from the corresponding ethanol fraction (130 mg.) derived from the same amount of material that was originally soluble in ammonium sulphate and which was most probably of smaller molecular size (Pusztai & Morgan, 1961*b*). Here again the mucopolysaccharide believed to be of smaller size was degraded by papain to give a larger yield of what is almost certainly a product of smaller size, as judged by solubility in ethanol. The importance of the narrowness of the molecular size range of the mucopolysaccharide entities contained in the starting preparation of the specific substance is well shown in the experiments made with A substance no. 401. According to the physical examination, this material, after very careful and thorough fractionation with both saturated ammonium sulphate at 60° and ethanol precipitation, contained a relatively narrow range of molecular sizes and, after exhaustive treatment with ficin and dialysis, could be separated quite sharply into mucopolysaccharide entities which were precipitated within the ethanol concentrations 48–53% and 58–66% respectively. No material precipi-

tated between 53% and 58% ethanol. The small amount (13 mg.) recovered from the 66% ethanol supernatant fluid contained lipid and other impurities from the original substance or from the reagents employed or from both.

The analytical figures in Tables 1–7 make it evident that as a result of the decomposition of the serologically specific mucopolysaccharides with ficin or papain, fragments are formed which are smaller in size and less active serologically. There is no evidence of any gross change in chemical composition when the amounts of the individual sugar components are determined in the decomposition products. There is one change, however, which is always found: the nitrogen content of the fragments is significantly lower than that of the material before incubation with the enzyme. In the preceding paper (Pusztai & Morgan, 1961c) it was shown that a limited number of peptide or ester bonds or both are broken during the digestion with ficin or papain. The results obtained in the present work substantiate the conclusion that a scission of peptide or ester bonds or both occurs, for it has been found that the fragments contain quantitatively less amino acid and thus account satisfactorily for the lower nitrogen value shown by these materials.

The results of the quantitative amino acid analyses given in Tables 11 and 12 indicate that the amino acid-containing part of the mucopolysaccharide macromolecule has a composition quite unlike that of any protein so far described. The high content of proline, 10–15% of the total amino acids, resembles that found in collagen, but otherwise the two materials are quite distinct. Similarly, the large amount of threonine and serine, which accounts for at least a third of the total amino acid in the two group A substances examined, is to be found in γ-globulin, but in few other proteins. The nearest in amino acid composition to the group mucopolysaccharides is the ovine salivary mucoprotein recently described by Gottschalk & Simmonds (1960).

The amino acid composition of the two main decomposition products shows significant differences from the original mucopolysaccharide when individual amino acids or groups of amino acids are compared. There is a net loss in amino acids and the heaviest loss for both decomposition products is on threonine and serine. The amount of individual amino acids found in the two main products makes it probable that the amino acids are not evenly distributed throughout the amino acid-containing structures in the original mucopolysaccharide. The bifunctional acidic or basic amino acids occur more frequently in the smaller of the decomposition products and therefore in that part of the amino acid-containing moiety in the original mucopolysaccharide which gives rise to this particular fragment. The results of some experiments made on columns of Dowex 50X2 to determine the nature and amount of any small decomposition products released during digestion that is materials not precipitated by 75% of ethanol, showed that nitrogenous materials, mostly peptides, but only small amounts of free serine, threonine and glutamic acid, were present. These free amino acids were probably secondary decomposition products of the peptides released during the initial phase of the decomposition of the mucopolysaccharide.

In conclusion, it would appear that the specific mucopolysaccharides are, as a result of the scission of a few peptide or ester bonds in the internal structure of the intact mucopolysaccharide macromolecule, degraded by ficin or papain into two main kinds of mucopolysaccharide fragments.

SUMMARY

1. The specific blood-group mucopolysaccharides have been incubated with crystalline ficin or papain and the products of proteolysis examined.

2. The mucopolysaccharides are broken down essentially into two kinds of macromolecular fragments and a small amount of diffusible peptide material.

3. The larger of the decomposition products possesses a few per cent only of the serological activity of the original substance when the activity is measured by the haemagglutination-inhibition test. The smaller of the two fragments has considerably less activity than that of the larger one.

4. The qualitative composition of the carbohydrate moiety of the decomposition products is identical with, and the quantitative composition close to, that of the original substance.

5. The quantitative amino acid composition of the products of the proteolytic decomposition of the specific mucopolysaccharides shows they are not identical with those of the original substance, or with each other. The evidence suggests that the amino acids are not evenly distributed throughout the amino acid-containing moiety in the original specific mucopolysaccharides.

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Macroglobulins

1. STUDIES ON THE ISOLATION AND PHYSICAL PROPERTIES OF PATHOLOGICAL MACROGLOBULINS

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On examination in the ultracentrifuge, human sera are shown to possess small concentrations of protein which sediment some two or three times faster (i.e. sedimentation coefficients in excess of 15s) than the more usual globulin components. These macroglobulin components normally account for up to 5% of the total protein, but Waldenstrom (1944) pointed out that certain pathological sera contain much larger quantities of a protein (or group of proteins) whose sedimentation behaviour is very similar to that of the normally occurring macroglobulin. More recent work (Wallenius, Trautman, Kunkel & Franklin, 1957; Deutsch & Morton, 1958; Kunkel, 1960) has confirmed that a group of differently sedimenting components rather than a single species is involved, and that electrophoretically both α- and γ-globulin types occur, though not necessarily in a single case. The comparison of the properties, both physicochemical and immunological, of normal and pathological macroglobulins presents a problem of some complexity, but additional difficulty is introduced when only imperfect separations of the macroglobulins from normal serum components are available.

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At the time the present work began, the great resolution obtainable through the use of chromatographic columns of ion-exchange cellulose materials had become apparent (Sober, Gutter, Wyckoff & Peterson, 1956; Sober & Peterson, 1958; A. Feinstein, P. Johnson & C. B. Richards, 1958, unpublished work). Such columns have been used to isolate from a macroglobulinaemic serum the macroglobulins and other globulins for detailed physicochemical characterization and comparison with other serum proteins. The immunological properties of the various protein fractions have been compared and are described in the next paper.

EXPERIMENTAL

Blood was collected by venepuncture and allowed to clot at room temperature. After 3½ hr. the serum was separated and had a protein content of 10·4 g./100 ml. No precipitation occurred on cooling it to 2°, but dilution with water (Sia test: see Martin, 1960) caused precipitation.

Isolation of macroglobulins

Fig. 1 shows the sedimentation patterns of the serum from the case of macroglobulinaemia (*b*) alongside normal serum sedimentation patterns (*a*). In the pathological serum it is clear that a group of macroglobulins (approx. 32% of total protein) rather than a single component is

involved. Serum from this case was used exclusively as the source of the proteins whose isolation and properties are described in this and the next paper. The serum was dialysed against aq. 1·64M-$(NH_4)_2SO_4$ at 2–4°; the supernatant liquid, containing most of the albumin and traces of S_7 globulin (a particular sedimenting species is denoted by the symbol S with, as subscript, the rounded value of its sedimentation coefficient) as well as macroglobulin (Fig. 1*d*),

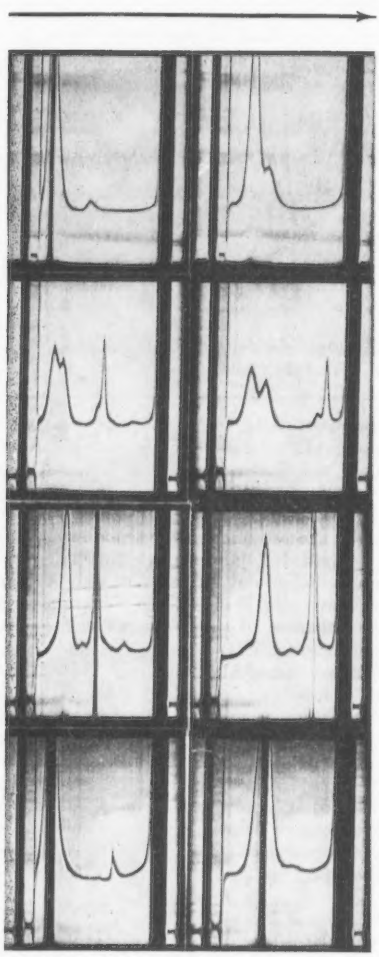


Fig. 1. Sedimentation patterns at comparable stages of sedimentation in phosphate–NaCl buffer, pH 7·8, I 0·1, for: (*a*) normal serum; (*b*) serum from a case of macroglobulinaemia; (*c*) precipitate from (*b*) with 1·64M-$(NH_4)_2SO_4$; (*d*) supernatant from (*b*) with 1·64M-$(NH_4)_2SO_4$. Rotational speed: 59 780 rev./min. Temperature: 16±1°.

was not further used for preparative purposes. The precipitate containing most of the S_7 globulins and macroglobulins (Fig. 1*c*) was redissolved in NaCl soln. up to the initial volume of the serum and was found to remain in solution at 2°. After dialysis against 5 mM-phosphate buffer (Na_2HPO_4, 5 mM) at pH 8·2, the solution was applied to a column of diethylaminoethylcellulose at 2–4°, prepared as described by Sober *et al.* (1956) and previously equilibrated against the same buffer solvent. Elution was performed by establishing a gradient of decreasing pH and increasing concentration of KH_2PO_4. The final concentration of KH_2PO_4 was 0·1M and the pH 4·7. Further protein was removed by elution with a solution containing 0·1M-KH_2PO_4 and 0·5M-NaCl, giving a total yield in excess of 90%.

Two such fractionations (I and II), employing columns 23 and 55 cm. long, with internal diameters of 3 and 3·5 cm. (retardation volumes of about 80 and 300 ml. respectively), were carried out on the proteins from 12 and 45 ml. of the original sera respectively. Sample volumes were 5 and 15 ml. respectively. $E_{280m\mu}$ of each sample, measured on a Unicam SP. 500 spectrophotometer, was plotted against sample (or tube) number to give the elution diagrams, which were then utilized to suggest pooling procedure. The 10 or 11 fractions thus obtained were concentrated by pervaporation or freeze-drying before further examination. Fractions 1–5 were stored as solutions at −20° for some 6 months before the ultracentrifugal studies were completed. In some such cases, changes in the state of aggregation became apparent. The main fractions, particularly 6.I and 9.I, were freeze-dried to the solid before storage at −20°. Fig. 2 shows the elution diagram for fractionation I, which differed from that of fractionation II only in superficial details.

Ultracentrifugal examination was carried out in a Spinco model E instrument equipped with schlieren phase plate. Unless otherwise stated the solvent employed was phosphate–NaCl buffer, pH 7·8, I 0·1, and solutions were exhaustively dialysed against repeated changes before examination. In correcting observed sedimentation coefficients to water at 20° (S^0_{20}), the partial specific volume assumed was 0·725 (Koenig, 1950; Dayhoff, Perlmann & MacInnes, 1952). Values of S^0_{20} extrapolated to zero protein concentration are denoted by $[S^0_{20}]$. Electrophoretic measurements were performed in a Perkin–Elmer apparatus equipped with a diagonal-schlieren optical system and the usual sectional U-tube of rectangular cross-section. Both ultracentrifuge and electrophoretic records were measured on a two-dimensional Pye travelling microscope; sedimentation coefficients (S) were then computed from plots of $\ln x$ against time at constant speed (x being distance from centre of rotation) and electrophoretic mobilities (u) from plots of boundary position (x) against time at constant current i $\{u = [(dx/dt)\,AK]/i$, where A is area of cross-section of tube and K is the specific conductivity of the protein solution$\}$.

Protein concentrations were determined from measurements of refractive index (n) in a Zeiss interferometer, assuming dn/dc = 0·187 ml./g. (Doty & Edsall, 1951) as for human γ-globulins. Since the actual value of dn/dc for macroglobulins was not determined (owing to the small quantities of purified material available) concentrations are quoted in terms of refractive-index differences. Occasionally, measurements of extinction were also used, for which

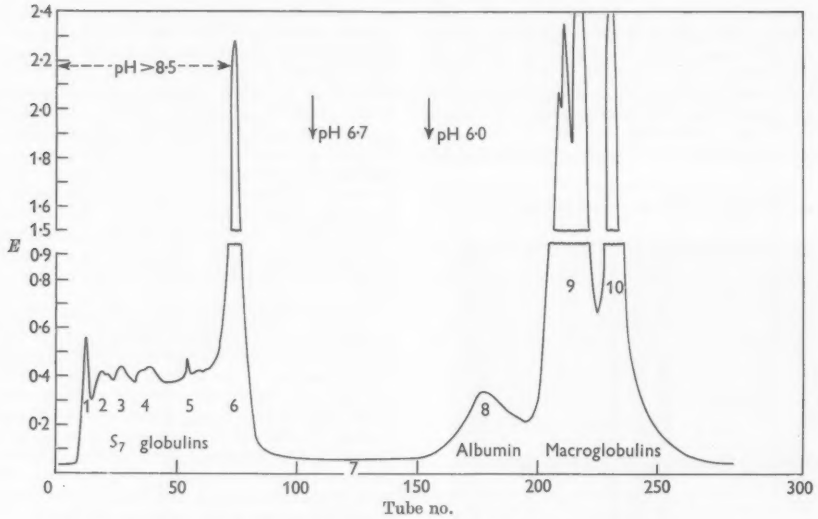


Fig. 2. Elution diagram for fractionation I of globulins from macroglobulinaemic serum on diethylaminoethyl (DEAE)-cellulose at 2–4°. Tube volume: 5 ml.

an empirical relation, $\Delta n = 1{\cdot}50 \times 10^{-4} E_{280}$, was found to apply at protein concentrations of up to 0·2 g./100 ml. In assessing the proportions of differently sedimenting components, peak areas at constant magnification and schlieren angle, corrected to the position of the meniscus, were utilized, equal refractive-index increments being assumed.

Materials

Buffer salts were of analytical or equivalent grade. 2-Mercaptoethanol was obtained from L. Light and Co. Ltd. and iodoacetamide from Hopkin and Williams Ltd.

The diethylaminoethylcellulose was obtained from L. Light and Co. Ltd. (type 40). It possessed approx. 1 m-equiv. of basic groups/g.

Bovine plasma albumin, used in the preparation of albumin solutions of varying concentration ($\leqslant 5$ g./100 ml.), was an Armour crystallized fraction (batch BF1670).

The carbohydrate content of various protein fractions was determined semi-quantitatively by the reaction with periodic acid–Schiff stain (Köiw & Grönwall, 1952). Equal weights of the proteins in solution were placed on Whatman 3MM paper and the red colours produced by the stain were compared visually. Total macroglobulinaemic serum and the isolated macroglobulin fractions gave definite positive tests for carbohydrate, but the slowly sedimenting fraction, 8.II, and the prominent S_7 fraction, 6.I, gave only weak and very weak reactions respectively.

RESULTS

The elution diagrams show that several minor components (1–5) and one major component (6) pass rapidly through the column with little or no retardation. After this group of components, a considerable volume of eluent passes through the column before the next broad peak (8) emerges, followed by a major complex of peaks. The fine structure of the complex was not reproducible between the two fractionations and it has therefore been divided, somewhat arbitrarily, into two components, 9 and 10 in fractionation I and an additional one (11) in II. Fig. 3 contains sedimentation patterns of the components from the two fractionations; ultracentrifuge examination was performed immediately for fractions 6.I, 9.I and 9.II, or in other cases at only a few days (at most) after elution from the column. Fractions 1–4 sediment very largely as single well-defined boundaries, with sedimentation coefficients of approximately 7s. Fractions 5 and 6 also contain similar components but, in addition, significant proportions of very slowly sedimenting material (1·25s) occur. In fractionation II, similar slowly sedimenting material appeared in a later fraction (8.II).

Fraction 8.I consisted largely of material sedimenting at a rate similar to that of albumin with traces of macroglobulins. Since the globulin fraction applied to the column was not reprecipitated, the presence of some albumin was not unexpected and, with an isoelectric point lower than the globulins, its position in the elution pattern was as expected. However, in fractionation II, little or no albumin was observed and fraction 8.II con-

sisted largely of material with $S^0_{20} = 1{\cdot}25$s. It would thus appear that albumin tends to displace the slowly sedimenting material.

The remaining fractions (9.I and 10.I, and 9.II, 10.II and 11.II) contained macroglobulins, and no difference between these fractions in their physical properties was apparent. Fractions 9.I and 9.II were the most abundant and were chiefly used in the work. Three main sedimenting components with S^0_{20} values (see below) of 17·0, 19·2 and 28·9s occurred in the proportions 5, 85 and 10%. Traces of slower (8, 11·5, 14s) and faster sedimenting components also occurred, for which accurate measurements are not available.

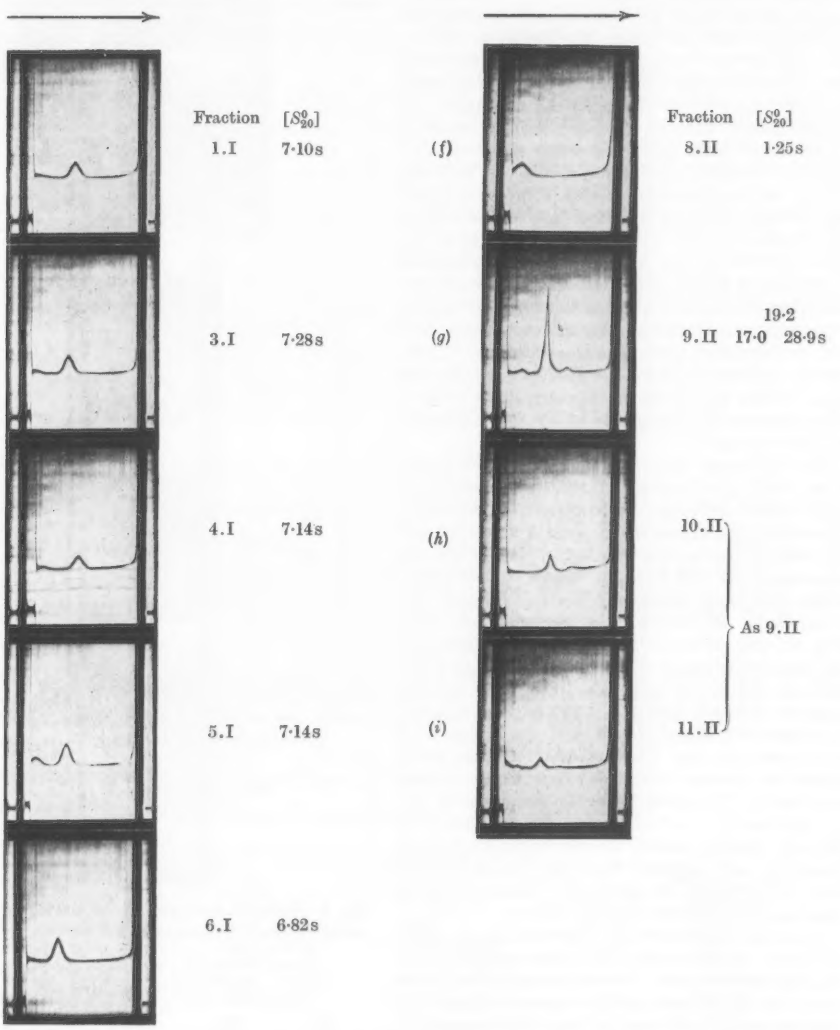


Fig. 3. Sedimentation patterns and data for representative fractions from two fractionations (I and II) of total globulins on DEAE-cellulose (cf. Fig. 2). Solvent: phosphate–NaCl buffer, pH 7·8, I 0·1. Speed: 59 780 rev./min.

Properties of the isolated macroglobulins

Since the differently sedimenting macroglobulin components did not separate on the chromatograms, the possibility arose that they formed an equilibrium system. In such a case the proportions of the components and measured properties should vary with the experimental conditions. Fig. 4 contains schlieren diagrams for experiments carried out at $17 \pm 2°$ at different total protein concentrations and pH values; no change in relative proportions was detected. Similarly, dilution of the original whole serum and of the total globulin fraction was without effect on the macroglobulin sedimentation pattern. Ultracentrifugal examination at 37° also gave the same sedimentation pattern. Further, except for species of 17·0 and 19·2s, the components resolve completely from one another, giving a true base-line between them which indicates lack of interaction. The difference, 17·0 to 19·2s, is not sufficient to allow complete resolution of the species involved, but the separation observed appears to progress normally during sedimentation. Further, the sedimentation data were extrapolated linearly in the usual way to zero protein concentration for the different components (Fig. 6); this would not be expected if the various peaks contained components of a rapidly reacting equilibrium system.

Since previous work (particularly of Wallenius *et al.* 1957) had indicated that macroglobulins could show differing electrophoretic properties, electrophoretic examination over a range of pH was carried out on fraction 9.II. Except for the experiment at pH 4·15, in which 0·2M-acetate buffer was used, phosphate–NaCl buffers, I 0·2, were used. Electrophoretic patterns for pH 8 (Fig. 5*a*) demonstrate the essentially homogeneous electrophoretic nature of the material and particularly the absence of more rapidly moving (α or β) material. The pH–mobility curve of Fig. 5*b* gives an isoelectric point of pH 5·9, and comparison with published data (e.g. Tiselius, 1937; Porter, 1960) shows the present macroglobulins to be typical γ-globulins. The electrophoretic homogeneity demonstrates that the differently sedimenting macroglobulin species possess the same electrophoretic character and suggests that the macroglobulins may be a family of chemically similar or even identical members as discussed below.

Since the macroglobulin fractions were stable mixtures of differently and independently sedimenting systems, they were examined in the ultracentrifuge at different protein concentrations. The sedimentation coefficients of the different species, corrected to water at 20°, were plotted as a function of the total initial protein concentration. Fig. 6 summarizes the results obtained for the fractions from both fractionations. Within experimental error, each sedimenting species gives its own linear plot for S^0_{20} against c (where c is total initial protein concentration in g./100 ml.). By the method of least squares, the parameters $[S^0_{20}]_{c=0}$, K and k of equations (1) and (2),

$$S^0_{20} = [S^0_{20}]_{c=0} - Kc \quad (1)$$

and

$$S^0_{20} = \frac{[S^0_{20}]_{c=0}}{1+kc} \quad (2)$$

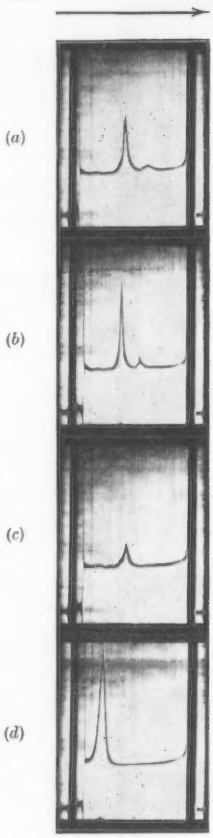
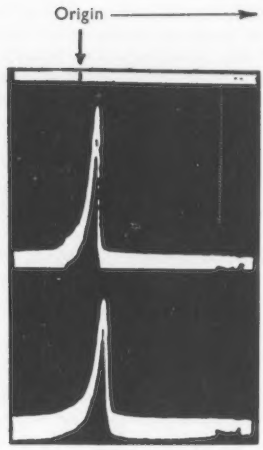
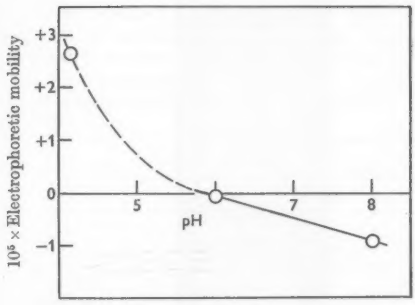


Fig. 4. Sedimentation patterns for macroglobulins (9.II) under various conditions at $17 \pm 2°$:

	Concn. of protein (g./100 ml.)	pH	Buffer
(*a*)	1·0	7·8	Phosphate, I 0·1
(*b*)	1·0	4·2	0·2M-Acetate
(*c*)	0·5	7·8	Phosphate, I 0·1
(*d*)	1·0	7·8	Phosphate, I 0·1 + 1% of mercaptoethanol



(a)



(b)

Fig. 5. Electrophoresis of macroglobulin fraction 9.II. (*a*) Ascending electrophoresis patterns in phosphate–NaCl buffer, pH 8·0, I 0·2. Time between exposures: 40 min. Potential gradient: 5·1 v/cm. (*b*) pH–mobility curve.

were computed (Table 1). The data of Fig. 6, and those derived from it, refer to a solution containing three main sedimenting species in different proportions. The S against c curve for the intermediate fraction, occurring to the extent of approx. 85%, has considerable significance. For the other two fractions only the intercept on the ordinate axis is important, since the slope in each of these cases is determined by the concentration of components other than itself.

For comparison, similar data for the earlier S_7 fractions and for bovine serum albumin (Harrington, Johnson & Ottewill, 1956; Johnson, 1957) are also provided with the corresponding S against c plots (Fig. 7). In all cases, the data fit linear plots within experimental error, but owing to the small quantities of some fractions available the concentration range was restricted.

The sedimentation behaviour of the dissociation products of macroglobulin fraction 9.II is also illustrated in Figs. 4 and 8. Dissociation was accomplished by dialysing for 50 hr. a solution of the macroglobulins containing 0·65 g./100 ml.

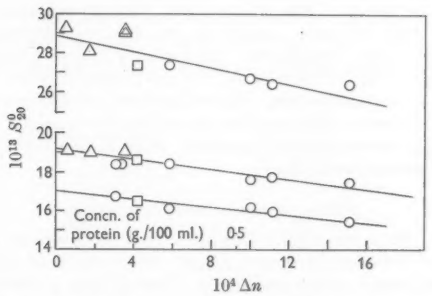


Fig. 6. Plots of S^0_{20} against total initial protein concentration (or Δn) for macroglobulin components from the different chromatographic fractions. ○, Fraction I, II, no. 9; □, fraction I, II, no. 10; △, fraction II, no. 11.

Table 1. *Sedimentation parameters*

Probable errors are given in parentheses.

Fraction	$[S^0_{20}]_{c=0}$	K (eqn. 1)	k (eqn. 2)	Concn. range (g./100 ml.)
Bovine serum albumin	4·45	0·32	0·072	0–1
6.I, 6.II, 7.II	6·82 (±0·01)	0·36 (±0·02)	0·052	0–1
1.I, 2.I	7·10 (±0·03)	0·89 (±0·1)	0·125	0–0·5
3.I	7·28 (±0·05)	1·97 (±0·3)	0·27	0–0·3
4.I, 5.I	7·14 (±0·01)	1·80 (±0·08)	0·25	0–0·3
9–11 I and II	17·0	1·94	0·12	0–1
	19·2	2·50	0·13	0–1
	28·9	3·88	$0·13_4$	0–1
9.II Dissociated	7·40	0·44	0·060	0–1

against 1% (by vol.) of 2-mercaptoethanol in phosphate buffer, I 0·1, pH 7·8 (Fig. 8*a*). Removal of the mercaptoethanol by further dialysis for 24 and 72 hr. against the buffer alone was followed immediately by ultracentrifugal examination (Fig. 8*b* and 8*c*). Dialysis against 0·01M-iodoacetamide in phosphate buffer was used to stabilize the dissociation products (Fig. 8*d*) whose S against c behaviour was then investigated (Fig. 7). As shown above, fraction 9.II contains the three main, differently sedimenting, macroglobulin components as well as traces of minor ones; the contrast with the well-defined, single, much more slowly moving peak of the dissociated material (Figs. 4*d* and 8) is striking. Thus apart from the small proportion (approx. 20%) of additional material that consistently appears on the trailing edge of the peak of the dissociated macroglobulin, the latter appears comparable in respect of homogeneity with the earlier S_7 fractions. The S against c extrapolation was linear and the sedimentation coefficients are consistently and significantly higher than for the naturally occurring S_7 fractions. The dissociated material (unstabilized) reaggregated very slowly after removal of mercaptoethanol by dialysis (see Fig. 8*b* and 8*c*); though polymeric forms certainly appeared, the higher, naturally occurring polymers (particularly those with $S^0_{20} = 19{\cdot}2, 28{\cdot}9$S) either did not appear or were in low concentration. The occurrence of the lower polymers confirmed that the minor components of the macroglobulin fractions were a part of the macroglobulin family.

Sedimentation of protein mixtures

Since macroglobulins are frequently observed sedimenting in serum, i.e. in the presence of considerable concentrations of albumin and globulin, a knowledge of the retarding effect of other proteins is important. Known mixtures of the macroglobulins with the well-defined S_7 fraction, 6.I, and with human serum albumin, were therefore prepared and the sedimentation behaviour of the various boundaries was investigated quantitatively (Table 2). In mathematical consideration of the sedimentation coefficients, the term kc in the denominator of equation (2) is replaced by $\Sigma k_i c_i$ (by analogy with Wallenius *et al.* 1957), where c_i is the concentration of the ith component at the boundary in question and k_i is the constant derived for it from equation (2) which expresses the variation in its sedimentation coefficient with its own concentration. Thus for the corrected sedimentation

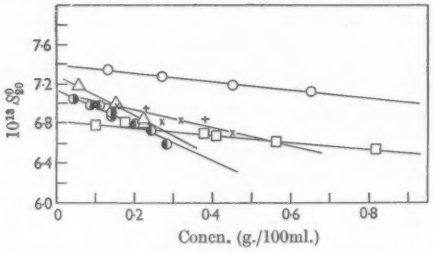


Fig. 7. Plots of S^0_{20} against concentration for different chromatographic fractions and for the iodoacetamide-stabilized product of macroglobulin dissociation (○). □, Fraction I, II, no. 6; +, ×, △, ◑ and ◐, fraction I, nos. 1–5 respectively.

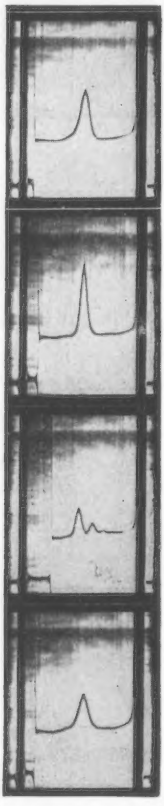


Fig. 8. Sedimentation patterns illustrating dissociation and reaggregation of macroglobulin fraction 9.II. Protein concentration: 0·6 g./100 ml. Buffer: phosphate–NaCl, I 0·1, pH 7·8. (*a*) After dialysis against buffer containing 1% of mercaptoethanol; (*b*) after further dialysis against buffer for 24 hr.; (*c*) after further dialysis against buffer for a total of 72 hr.; (*d*) after (*b*), dialysed against 0·01M-acetamide for 24 hr.

coefficient (S^0_{20}) of a component at finite concentrations of itself and other components, equation (2) becomes

$$S^0_{20} = \frac{[S^0_{20}]_{c=0}}{1+\Sigma k_i c_i}. \qquad (3)$$

Values of k_i for fraction 6.I and bovine serum albumin have been given in Table 1.

Table 2 contains summarized results for the various mixtures. Column 3, headed S^0_{20} (obs.), contains values of the sedimentation coefficient corrected to water at 20° by the usual expression

$$S^0_{20}\text{ (obs.)} = S \times \frac{\eta_t}{\eta^0_{20}} \times \frac{1-\bar{v}\rho^0_{20}}{1-\bar{v}\rho},$$

where S is the experimentally observed coefficient at temperature t in buffer solvent of viscosity η_t and density ρ, and η^0_{20} and ρ^0_{20} are the viscosity and density of water at 20°. Thus the contribution of the slower sedimenting protein to the density and viscosity of the medium through which the faster sedimenting protein moves is not considered at this stage. Column 4 contains values of $[S^0_{20}]_{c=0}$ calculated from equation (3), and column (5) those determined by extrapolation of S against c curves for the pure proteins. Just as the calculation of $[S^0_{20}]_{c=0}$ from equation (2) for a single component at finite concentration includes the effect of increased viscosity and density of the medium, so does that from equation (3), and no correction for change in properties of the medium arising from the presence of slower sedimenting components is required. Such a correction is involved in the $\Sigma k_i c_i$ term.

It is of interest, however, to calculate separately the retardation of the faster sedimenting material caused by the increase in density and viscosity of the medium due to slower components. If such a correction procedure were valid, the corrected sedimentation coefficient of the faster sedimenting material must correspond to a finite concentration of itself rather than $[S^0_{20}]_{c=0}$. Viscosity and density measurements over the necessary concentration range are not available for the various fractions, but were performed for bovine serum albumin, which in its physicochemical properties strongly resembles human serum albumin. Graphs of relative density and viscosity were constructed for concentrations approaching 5 g./100 ml., and were used to compute the appropriate correction factors. The corrected S^0_{20} values are contained in column (6).

Changes in chromatographic fractions on storage

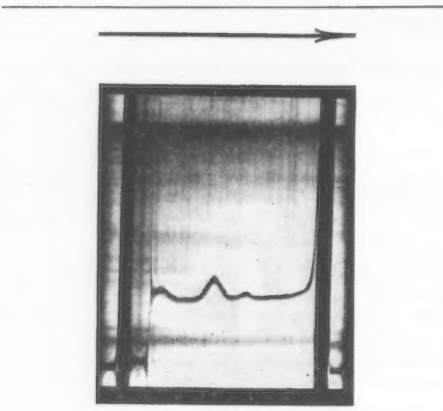
Whilst fractions 1–6, shortly after elution from the chromatographic column, consisted largely of

Table 2. *Sedimentation of protein mixtures*

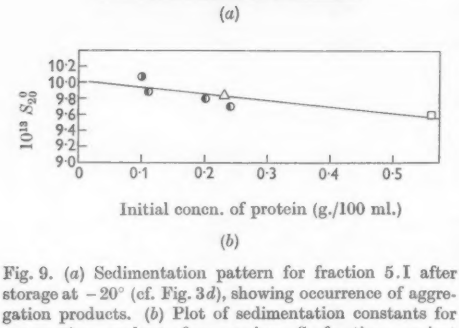
Components	Concn. (g./100 ml.)	S^0_{20}(obs.)	$[S^0_{20}]_{c=0}$ Calc. (eqn. 3)	$[S^0_{20}]_{c=0}$ Obs.	S^0_{20}(corr.)*
9.II	0·56	17·23	19·0	19·2	—
		27·15	30·4	28·9	—
6.I	0·61	6·8	7·0	$6{\cdot}8_2$	—
9.II	0·56	15·52	16·9	17·0	—
		17·52	19·0	19·2	—
		26·78	29·2	28·9	—
6.I	$0{\cdot}31_5$	6·83	7·0	$6{\cdot}8_2$	—
Macroglobulin (II)	Traces	14·77	19·15	19·2	18·1
Albumin	4·2	—	—	—	—
Macroglobulin (I)	Traces	16·35	19·4	19·2	18·9
Albumin	2·7	—	—	—	—
Macroglobulin (II)	0·26	14·87	18·0	19·2	17·0
		21·67	26·1	28·9	24·8
Albumin	2·52	—	—	—	—
Macroglobulin (I)	$0{\cdot}43_7$	17·63	19·0	19·2	—
	0·06	27·28	29·4	28·9	—
Globulins	0·31	6·85	6·95	(7·0)	—
Macroglobulin (I)	2·19	12·59	17·1	17·0	—
	0·30	18·20	25·7	28·9	—
Globulins	1·45	6·08	6·50	(7·0)	—
Albumin	$0{\cdot}12_3$	4·30	4·34	4·46	—

* Calculated by using increased density and viscosity of solvent due to presence of slower sedimenting components.

material with $S^0_{20} \approx 7$s with no significant proportion of faster material, after storage at $-20°$, both in solution and in the dry solid state, appreciable proportions of faster material were completely resolved (Fig. 9) from the main component. The new components must have been formed from the S_7 components, but if the change in pattern represented the approach to equilibrium, then, during any single sedimentation experiment, equilibration was too slow to affect sedimentation patterns. The S values computed could thus be taken to be quantitatively related to molecular weights. To obtain an approximate estimate of the sedimentation coefficient of the new products at zero concentration, the observed corrected S^0_{20} values were plotted (Fig. 9*b*) against total initial protein concentration for the different fractions. When the differences between the various S_7 fractions are considered the plot is surprisingly good, and indicates a $[S]^0_{20}$ value of approx. 10s. This may indicate dimerization of the S_7 molecules, with relatively minor changes in frictional ratio.



(*a*)



(*b*)

Fig. 9. (*a*) Sedimentation pattern for fraction 5.I after storage at $-20°$ (cf. Fig. 3*d*), showing occurrence of aggregation products. (*b*) Plot of sedimentation constants for aggregation products from various S_7 fractions against total initial protein concentration. Fraction I, no. 3 (△), no. 4 (◑), no. 5 (●) and no. 6 (□).

DISCUSSION

The macroglobulins described in this paper behave electrophoretically as typical γ-globulins. This supports the suggestion of Peterson & Sober (1960) that the slow elution (under the experimental conditions) of the macroglobulin relative to γ-globulins is due to the increased adsorption arising from high molecular weight. Elution of the macroglobulin was, in fact, very similar to that of albumin, so that for chromatographic purposes the increased adsorption due to higher-molecular-weight of the macroglobulins was more than equivalent to a lowering of the isoelectric point by one pH unit.

The well-defined character of the earlier S_7 fractions from the chromatogram probably arises also from the fact that higher states of aggregation, which form on storage, are held back on the column so that the 'monomer' only is eluted in the early stages. The aggregated forms would correspondingly elute at a later stage, possibly with the macroglobulins, and could thus give rise to contamination. Since the aggregated forms may be in equilibrium with the 'monomer' under normal conditions, during elution they would at least partially revert to monomer. The misleading impression would thus be obtained that the monomeric form was being partially retained after the first group of fractions containing it had been completely eluted.

From the nature of the processes employed (precipitation, chromatography and freeze-drying), it is clear that minor serum components which are normally undetected may be detected and concentrated. This probably explains the slowly sedimenting component (fraction 8.II, Fig. 3), occurring in both fractionations, which is invisible in the sedimentation patterns of the original serum or separated globulin fraction. This material is almost certainly of low molecular weight and its elution at the later stages probably indicates a low isoelectric point ($< 4{\cdot}5$). It did not specifically affect the sedimentation of other protein fractions and in its chromatographic behaviour, as mentioned above, it appeared to be displaced by albumin.

Although the existence of a range of electrophoretic and associated properties within the γ-globulins has long been accepted (e.g. Sharp, Cooper & Neurath, 1942; Alberty, 1948), it is important to establish whether or not the fine structure of the elution diagram is meaningful. Previous work (Fahey & Horbett, 1959) tended to show definite electrophoretic differences between the different fractions; the present work has shown the occurrence of significant differences in sedimentation behaviour. Not only are differences in the extrapolated sedimentation coefficients, $[S^0_{20}]_{c=0}$, apparent, but the slopes of the linear S against c

plots appear to vary with the latter. In view of the high precision of sedimentation-velocity measurements, it seems that real separations of the different S_7 species are performed by the chromatographic column. However, the different S_7 fractions have the common property of aggregating, probably to the dimer, on storage at $-20°$ as the dry solid or in solution in the frozen condition. This property has not been attributed to S_7 material previously, and it may indicate that the S_7 fractions of the particular serum used have abnormal properties.

The macroglobulins discussed in this work are associated with a pathological condition, and for this reason are not necessarily comparable with normal macroglobulins. Except when reducing agents were present, the macroglobulins sedimented as several distinct species both in the original serum and in the purified form (cf. Martin, 1960). On the other hand, electrophoretically, the absence of α-globulin-type material is noteworthy as well as the typical γ-globulin, single-peak electrophoretic behaviour. The group of differently sedimenting macroglobulins with common electrophoretic properties provides a further example of the general statement that electrophoretic properties are largely independent of state of aggregation (e.g. Johnson & Naismith, 1953, 1954).

The subdivision of the macroglobulins by the column into two main and various subsidiary peaks was not reflected in any differences in sedimentation behaviour. However, a systematic and careful electrophoretic comparison was not performed, and electrophoretic differences, which would explain the slightly different chromatographic behaviour, may well exist.

As indicated by previous work (see, for example, Kunkel, 1960), the macroglobulin components show no sign of dissociation or aggregation with changing pH, protein concentration or temperature. Kunkel (1960) has shown that the distribution among the differently sedimenting members is similar in different cases of macroglobulinaemia; the present case, with the bulk of the material sedimenting with $[S^0_{20}] = 19{\cdot}2$s, superficially fits into this scheme. Thus in general some 70–90% of the macroglobulin occurs as the component with $[S^0_{20}] \approx 19$s, with 10–20% of $[S^0_{20}] \approx 25$–29s and smaller quantities of other components. The occurrence of carbohydrate in the macroglobulins is also common to many other cases (e.g. Kunkel, 1960; Martin, 1960). However, on careful examination differences are apparent. Thus even allowing for inaccuracy in the sedimentation coefficients, it is apparent that there is considerable variation in the sedimentation coefficients for the main species, e.g. 17·8–19·9s and 25·0–31·0s for prominent components (Deutsch & Morton, 1958; Kunkel, 1960; Martin 1960), and certain components occur in some cases which are absent in others. Thus the component with $[S^0_{20}] = 17{\cdot}0$s occurring in the present work is absent in other published work (e.g. Deutsch & Morton, 1958; Kunkel, 1960; Metais, Kovacs-Jung & Daune, 1960), and components sedimenting faster than that of $[S^0_{20}] = 28{\cdot}9$s are largely absent from the present work but have been consistently reported elsewhere. Thus the different macroglobulin systems seem to resemble one another only superficially.

In response to sulphydryl compounds (cf. Deutsch & Morton, 1958), however, the differently sedimenting species of macroglobulins all dissociate completely, to give mainly the same type of dissociation unit in view of the well-defined sedimenting boundary (Fig. 8*a*). There is a small proportion (about 20%) of more slowly sedimenting material, visible on the trailing edge of the peak, and this persists after stabilization of the product by iodoacetamide. No accurate sedimentation coefficient for this material could be obtained, but after stabilization with iodoacetate its sedimentation constant was approximately 5s. On removal of mercaptoethanol by dialysis (Fig. 8*b*), the slower-sedimenting dissociation products rapidly disappear, by contrast with the bulk of the dissociated material, which reaggregates only very slowly and incompletely. A small proportion of the macroglobulins appears to be broken down into units which differ from the main dissociation products not only in their slower sedimentation but also in their more rapid rate of recombination. The similarity of the recombination products with the minor components, seen in the isolated macroglobulin, suggests that these minor components are not impurities but, rather, lower states of aggregation of the macroglobulin system. The electrophoretic identity of the differently sedimenting components supports this view.

The main bulk of the dissociation product gave a good linear S against c plot that was significantly displaced above the corresponding lines for all of the earlier S_7 materials. That this displacement was not due to the uptake by the protein of iodoacetamide was shown by the fact that the sedimentation coefficient of unstabilized dissociated material, immediately after removal of mercaptoethanol by a short dialysis, fell close to the same line. Thus the dissociation products of the macroglobulins are not normal S_7 materials. Not only do they possess chemically active groups responsible for the polymerization into macroglobulins, but the molecules must be different in size or in shape (or both) to account for the differences in sedimentation.

A linear dependence of sedimentation coefficient on protein concentration satisfactorily explains experimental results. It was to be expected that

K ($[S^0_{20}]$ k) values would increase with increasing sedimentation coefficient and molecular weight, but no trend in k is apparent.

In considering the sedimentation of mixtures, perhaps the most obvious procedure in correcting to standard conditions the observed sedimentation of a fast component in the presence of slower-sedimenting materials would be based upon the viscosity and density changes arising from the presence of the slower. However, such a procedure, though not rigorously applied here, appears to 'undercorrect' considerably, even when it is realized that the corrected sedimentation coefficients (Table 2, column 6) refer to a finite concentration of the fast component. On the other hand, equation (3), particularly for the lower protein concentrations (< 2 g./100 ml.), yields values of $[S^0_{20}]_{c=0}$ from measurements in protein mixtures which are in good agreement with those obtained from single-protein systems. At the higher protein concentrations (> 2 g./100 ml.) the procedure also tends to undercorrect, but more data are required to substantiate this point. Equation (3) implies that the faster component is retarded by the slower to the same extent as the latter retards itself at the same concentration, i.e. specific interactions between the components are absent.

SUMMARY

1. The fractionation, by chromatography on diethylaminoethylcellulose, of the total serum globulins from a case of macroglobulinaemia has been described. By the use of a gradient of increasing salt concentration and decreasing pH, a series of globulins, distinguishable but all sedimenting at about 7s, was first eluted; macroglobulins emerged much later as a complex of peaks. The different peaks of the complex did not differ in their sedimentation behaviour.

2. The macroglobulin fractions all contained three main stable sedimenting species (85% with S^0_{20} of 19·2s, 10% of 28·9s and 5% of 17·0s) and traces of minor components, the sedimentation pattern being independent of total concentration, pH and temperature over considerable ranges. The differently sedimenting species were typical γ-globulins electrophoretically, with an isoelectric point at pH 5·9, and contained carbohydrate.

3. Plots of corrected sedimentation coefficient (S^0_{20}) against total initial protein concentration were linear and the extrapolated values at zero concentration differed from those reported for other macroglobulins.

4. The different macroglobulin species were converted by 2-mercaptoethanol into a much slower and more homogeneously sedimenting material. On stabilization with iodoacetamide, the detailed sedimentation behaviour was shown to be significantly different from that of the S_7 globulins from the same serum. Reversal of the dissociation was slow and incomplete, slower-sedimenting species comparable with the originally occurring minor components now occurring more prominently. This evidence, and the electrophoretic identity of the differently sedimenting macroglobulin species, suggest that the macroglobulins in this pathological case consist mainly of a number of different states of aggregation of the same monomeric unit.

5. From sedimentation-velocity experiments on mixtures, it was found that the faster sedimenting components were retarded by slower material to the same extent as the slower retarded itself from its infinite dilution value. On the other hand, the observed retardation of the faster components was greater than would be calculated from the increments in viscosity and density arising from the presence of the slower.

We are grateful to Dr d'A. Kok and Dr D. Whitmore of the University Departments of Medicine and Pathology for providing the macroglobulinaemic serum. Dr Whitmore also performed the semi-quantitative carbohydrate estimations. To Mr N. Buttress we are indebted for the careful performance of ultracentrifuge work. A.A. is further indebted to the British Council for a Fellowship held during the performance of the work.

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Macroglobulins

2. IMMUNOCHEMICAL STUDIES ON ISOLATED PATHOLOGICAL MACROGLOBULINS

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Albert & Johnson (1961) described the isolation by chromatography of the macroglobulins and other protein fractions from the serum of a single case of macroglobulinaemia. The same macroglobulin and accompanying protein fractions have been further characterized and compared by immunochemical studies, which are now reported. Previous work along these lines has given conflicting results. Thus Morton & Deutsch (1958), on the basis of cross-reactivity and absorption experiments, conclude that several pathological macroglobulins 'do not contain any immunological determinants lacking in normal human serum' and that the iodoacetamide-stabilized dissociation products as well as the reaggregated molecules possess the same antigenic groupings. However, from similar experiments, Franklin & Kunkel (1957) consider that though 7s and 19s components of γ-globulin cross-react, they are not immunologically identical. Similarly Korngold & Van Leeuwen (1957), regarding pathological macroglobulins as immunologically related to normal (γ_1-)-macroglobulins and 7s γ-globulins, report the preparation of antisera to macroglobulins, which by absorption with normal γ-globulins can be made specific for homologous macroglobulin. In substantial agreement with this view, Fahey & Horbett (1959) show that 18s and 7s γ-globulins give rise to distinct and separate precipitation lines when allowed to diffuse against anti-(whole γ) serum.

The different views held might conceivably arise from the different states of purity achieved by different workers for both macroglobulins and 7s components. Results on chromatographically isolated fractions thus become of considerable interest.

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MATERIALS AND METHODS

Serum from a single case (P) of macroglobulinaemia was fractionated on diethylaminoethylcellulose as described by Albert & Johnson (1961) and the various macroglobulin and 7s globulin fractions were utilized in both gel-diffusion (Ouchterlony, 1949) and immunoelectrophoresis (Grabar, 1958) studies.

The macroglobulins used, in both native and dissociated form, were mainly fractions 9 from both fractionations (I and II). Fractions 10.I and 11.II were very similar in composition, but since much smaller quantities were available and they had been less thoroughly characterized physicochemically, they were not much utilized in this section of the work. Of the 7s γ-globulin fractions (all from fractionation I), fraction 6 (γ-6) was mainly used but γ-2, γ-3 and γ-4 were also studied. The different proteins were used in phosphate–NaCl buffer, I 0·1, pH 7·8, at the following concentrations (g./100 ml.): macroglobulins (native and dissociated) 0·65–0·70; 7s globulin fractions (except γ-6), 0·2–0·4; γ-6, 1·0. In addition to the P serum and derived fractions, a small sample of a further serum (S) containing macroglobulins was made available by Dr R. Arden Jones of Newmarket General Hospital. Some months earlier, the patient (S) had shown a much higher content of macroglobulin ($\approx$47%), but, after treatment, the macroglobulin fraction now accounted for some 11% of the total protein. The following antisera were kindly provided by Professor J. R. Marrack and Dr A. Kelus. Rabbit antiserum against macroglobulin 9.II (AM) was prepared by a single multiple subcutaneous injection together with Freund's adjuvant. Anti-γ-globulin serum (Aγ) was prepared in a rabbit by injecting some of the animal's own red cells to which human γ-globulin (contaminated with other serum proteins) had been coupled by bisdiazotized benzidine. The rabbit received ten such injections intravenously.

A further anti-γ-globulin serum (Aγ_T) was prepared in a

rabbit from pooled human sera from patients with cirrhosis of the liver. The γ-globulins were precipitated with thymol, washed, mixed with Freund's adjuvant and injected subcutaneously.

A rabbit antiserum against whole human serum (AW) was prepared as follows. *Salmonella typhi* were coated by exposure to a convalescent typhoid serum, washed and injected intravenously. Somewhat surprisingly the antiserum produced reacted to all the main serum components.

The agar plates for double diffusion were prepared from 1% New Zealand agar in 0·9% NaCl soln. containing 0·1% of sodium azide. The wells, of 5 mm. diam., were bored at the corners and centre of a regular hexagon of side 10 mm. with the help of a jig. After filling the wells with the appropriate solutions, development was allowed to proceed in a saturated atmosphere at room temperature ($16\pm2°$) for some 18–20 hr., when the precipitation lines were readily visible. For permanency, the unchanged protein was dissolved by extensive washing in 0·9% NaCl soln. The plates were then dried at 37° after gently squeezing filter paper on the whole surface.

The lines were stained with 0·2% Ponceau S in 3% trichloroacetic acid or by Naphthalene Black in methanol–water–acetic acid (50:50:10, by vol.). The latter dye gave rise to more general background staining but was the more successful for the detection of faint precipitation lines.

Immunoelectrophoresis was performed in a gel formed from 1% of agar in 0·06 M-boric acid–NaOH buffer, pH 8·25, containing 0·1% of sodium azide. Parallel slots, 2·5 mm. wide and 7 cm. long, were cut 1·5 cm. apart in a rectangular plate (9 cm. × 7·5 cm.) and cylindrical wells, 2·5 mm. diam., were bored centrally between them and half-way along the length of the slots. A potential of 220 v (d.c.) was applied between platinum-foil electrodes immersed in the above-mentioned boric acid buffer. Washing and staining procedures were as already described for gel diffusion.

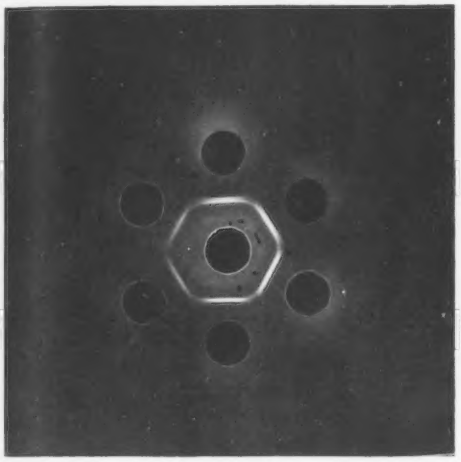
RESULTS

The macroglobulin (9.I or 9.II) gave two or possibly three precipitation lines against its own serum (Plate 1, *A*, *C*); the γ-globulin fractions (3, 4, 5 and 6) gave no detectable response against the same antiserum (Plates 1, *B*; 2, *A*). The absence in the macroglobulin fractions of material sedimenting at about 7 s (S_7 material) and of macroglobulin in S_7 fractions was thus strongly indicated, demonstrating the success of the column fractionation (see below, however). The slowly sedimenting fraction 8.II gave a feeble response against anti-M serum, indicative of slight impurity of macroglobulin.

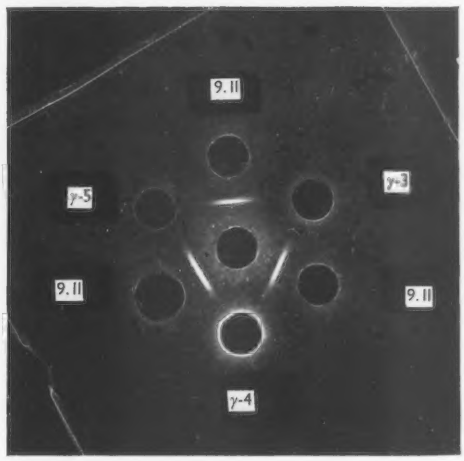
Dissociated macroglobulin gave a less well-defined precipitation pattern than the parent macroglobulin (Plate 1, *C* and *D*), a precipitation line (near the antigen well) being missing. The one broad band occurring gave a reaction of partial identity with 9.II (Plate 2, *A*), in conformity with previous observations (Korngold & Van Leeuwen, 1957). In Plate 1 (*C*) the lower well containing dissociated macroglobulin had not been freed from excess of mercaptoethanol. The effect of rapidly diffusing mercaptoethanol on 9.I can be seen by comparing the lower right with upper left well, which contains 9.I in the absence of reducing agents. No such effect occurs when the latter are removed and the protein is stabilized by iodoacetamide.

In Plate 2 (*A*) and (*B*), a comparison of the precipitation patterns of macroglobulin with AM and with the two different anti-γ-globulin sera is made. In interpreting these patterns it is to be recalled that the γ-globulins against which the antisera were prepared undoubtedly contained macroglobulins and probably traces of β- and α-globulins as well as the main γ component. The occurrence of an antibody common to AM and anti-γ sera is clear, but an extra precipitation line with the latter is also clearly visible. This line is apparently to be identified with the strong line occurring between γ-6 and anti-γ serum (Plate 2, *B*), and it is of note that dissociated macroglobulin gives an apparently identical line against anti-γ serum. It would therefore appear that the macroglobulin contains an impurity of S_7 material in low concentration which was not antigenic when injected with the macroglobulin into the rabbit (since γ-6 does not react with AM sera). When the antibody to this material is otherwise stimulated (as by injection at higher concentration in whole γ-globulin), the S_7 impurity, occurring alongside native or dissociated macroglobulin, reacts with it to give the extra precipitation line.

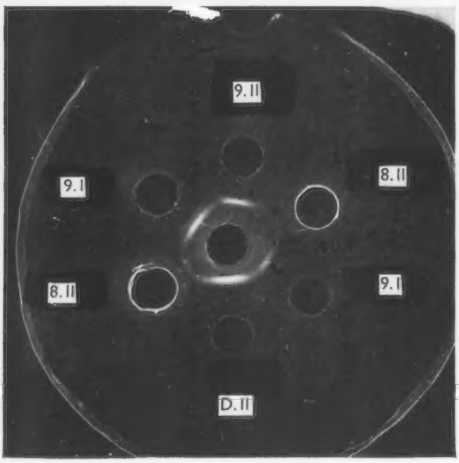
Immunoelectrophoresis was undertaken to investigate these reactions further (Plate 2, *D*). The macroglobulins were placed in the wells, and subjected to electrophoresis alongside the total globulins (see Albert & Johnson, 1961) from P serum and total serum from a different case (S) of macroglobulinaemia. In the troughs were rabbit antisera against macroglobulins, γ-globulins and total human serum. The precipitation-line system due to the macroglobulins accounts for only a small portion of that occurring between total γ-globulins and total human serum. Against AM sera, the macroglobulins (both 9.I and 9.II) give rise to two precipitation lines in the γ-globulin region, to be identified with those of Plate 1 (*A*), and an extra line near the origin against Aγ serum. The antigen responsible for this line is probably of somewhat higher mobility at pH 8 than those responsible for the main macroglobulin precipitation lines, but no exact electrophoretic description can be made from motion in an electrical field in a gel medium. The extra line here is most probably to be identified with that observed in double-diffusion plates against anti-γ sera (Plate 2, *A*, *B* and *C*), where it has been shown that the antigen responsible is related to γ-globulin. The occurrence of



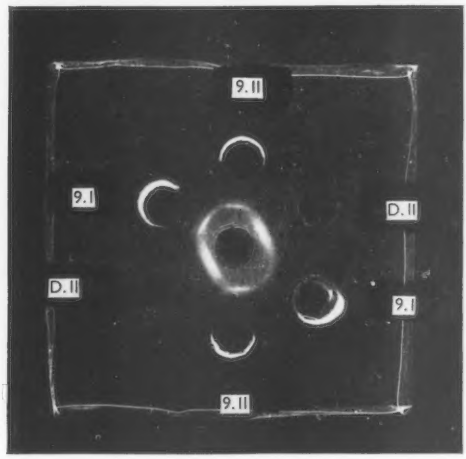
(*A*) Centre well: anti-macroglobulin (AM) serum. Top well: Macroglobulin (9.II) at concentration (*c*) 0·65 g./100 ml. Clockwise from the right of the top well, other wells contain macroglobulin at concentrations 3/4 *c*, 1/2 *c*, 1/4 *c*, 1/6 *c* and 1/7 *c*.



(*B*) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.II) and various 7s chromatographic fractions.



(*C*) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.II), dissociated macroglobulin (D.II) (containing mercaptoethanol) and fraction 8.II.



(*D*) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.I and 9.II) and dissociated macroglobulin (D.II).

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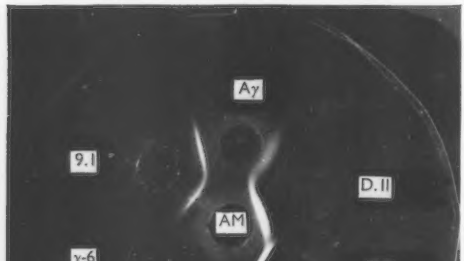
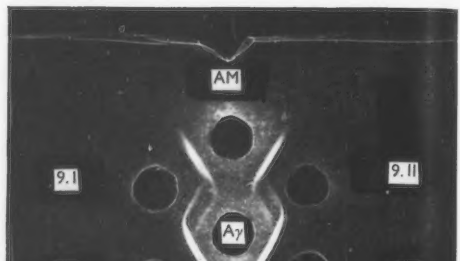
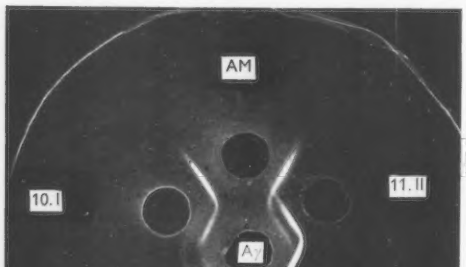
(*Facing p.* 670)



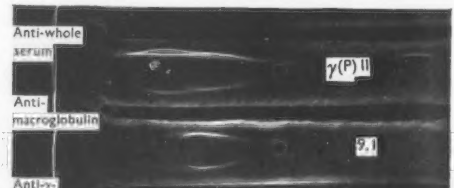
(*A*) Top and bottom wells: anti-γ-globulin serum ($A\gamma_T$). Centre well: anti-macroglobulin (AM) serum. Other wells contain macroglobulin (9.I and 9.II), dissociated macroglobulin (D.II) and the 7s fraction (6.I).



(*B*) Top well: anti-macroglobulin (AM) serum. Centre well: anti-γ-globulin serum (Aγ). Other wells contain macroglobulin (9.I and 9.II), dissociated macroglobulin (D.II) and the 7s fraction (6.I).



(*C*) Top well: anti-macroglobulin (AM) serum. Centre well: anti-γ-globulin (Aγ) serum. Other wells contain macroglobulin fractions (9.I, 10.I, 11.II) and 7s fraction (6.I).



(*D*) Immunoelectrophoresis. Solutions of macroglobulins (9.I and 9.II), total γ-globulins (from same P serum) and total serum proteins from S serum (see text for details) were placed in various wells and subjected to electrophoresis. Troughs were then filled with one of the following antisera: anti-macroglobulin, anti-γ-globulin (Aγ) and anti- (total) human serum.

such material in small concentration occurring to the extent of up to 5% of the total protein alongside the main macroglobulin is not in any sense ruled out by previously published sedimentation diagrams. The absence of the corresponding line between the total globulins of P serum and AM serum confirms the absence in the AM antiserum of the relevant antibody (see above), which apparently requires higher antigen concentrations for its formation than are present in the macroglobulin preparation.

The whole serum (S), while showing certain lines common to the P macroglobulins against AM serum, shows definite differences and its precipitation lines are relatively weak, indicating relatively low concentration of the macroglobulins. The ultracentrifuge gave a similar indication about concentration, but no qualitative differences between the macroglobulins occurring and those of P serum.

In Plate 2 (*C*), chromatographically different fractions containing macroglobulin (9.I, 10.I, 11.II) are compared alongside γ-6 in their reactions with AM and Aγ antisera. Although the different macroglobulin fractions all give the typical macroglobulin line and the extra line against Aγ, it is clear from the strengths of the lines that 10.I and 11.II both contain the γ-6 type of impurity in higher concentration than 9.I.

DISCUSSION

The results confirm the great resolution possible in the chromatography of proteins on diethylaminoethylcellulose. Thus the major macroglobulin fractions contain 7s γ-globulin impurity in such small amounts that the latter did not stimulate antibody production when injected into a rabbit in the macroglobulin fraction. That the impurity did occur, however, in amounts too small to be observable in the ultracentrifuge, was shown by the reaction of these macroglobulin fractions with anti-whole-γ-globulin sera, when a precipitation line, additional to those occurring with anti-macroglobulin sera, occurred. Such impurity also occurred in larger concentration in the minor macroglobulin fractions.

Similarly, if macroglobulin occurred in 7s globulin fractions, it was in too low a concentration to give rise to a visible precipitation line with anti-macroglobulin sera. These conclusions are in substantial agreement with those of Fahey & Horbett (1959), who chromatographically isolated 7s and 18s γ-globulins and showed that they gave rise to distinct and different precipitation bands against anti-whole-γ-globulin. The claim by Korngold & Van Leeuwen (1957) to have produced antisera specifically against macroglobulins is also in accord with the above conclusions.

Reports of cross-reactivity and antigenic identity between macroglobulins and 7s γ-globulins may have arisen from imperfect separations, based usually upon solubility differences between the two types of molecule.

SUMMARY

1. The main chromatographically separated macroglobulins gave two well-defined precipitation lines against anti-macroglobulin sera produced in the rabbit.

2. Chromatographically separated γ-globulin fractions did not give detectable precipitation lines against the same antisera.

3. Against anti- (whole γ-globulin) sera (Aγ), macroglobulin fractions gave rise to the same lines as against anti-macroglobulin sera and an extra weaker line related to that produced when γ-globulin fractions reacted with Aγ sera. Apparently a γ-globulin-type impurity occurred in the main macroglobulin fractions at a concentration such that it did not, on injection, stimulate the formation of its respective antibody. Subsidiary macroglobulin fractions contained such an impurity in higher concentration. The absence in the anti-macroglobulin serum of the antibody corresponding to the 'extra' precipitation line was shown by absence of the line when whole γ-globulins diffused against anti-macroglobulin sera.

We are grateful to Professor J. R. Marrack and Dr A. Kelus, of the Department of Pathology, Cambridge, for the various antisera used in this work, to Dr d'A. Kok and Dr D. Whitmore of the Departments of Medicine and Pathology for the macroglobulinaemic serum (P) and to Dr R. Arden Jones of Newmarket General Hospital for serum (S). A.A. is also indebted to the British Council for a Fellowship held during the performance of the work described.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **408th Meeting** *of the Society was held in the Department of Biochemistry, University of Oxford, on Thursday and Friday,* 13 *and* 14 *July* 1961, *when the following papers were presented:*

COMMUNICATIONS

Two Enzymic Mechanisms for the Methylation of Homocysteine by *Escherichia coli*

By M. A. Foster, Genoveva Tejerina and D. D. Woods. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Ultrasonic extracts of *Escherichia coli* PA 15 can synthesize methionine from homocysteine and serine when the folic acid cofactor is tetrahydropteroyltriglutamate or an extract of heated organisms (EHC); tetrahydropteroylmonoglutamate inhibits competitively both tetrahydropteroyltriglutamate and EHC, the inhibition being overcome by cobalamin (Szulmajster & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960; Jones, Guest & Woods, 1961). However, with ultrasonic extracts of *E. coli* 121/176 (a methionine/cobalamin auxotroph) cobalamin is required with EHC, tetrahydropteroyltriglutamate or tetrahydropteroylmonoglutamate. Cobalamin acts by prior conversion into a complex factor (Kisliuk & Woods, 1960; Guest & Woods, 1960), which has been purified from strain PA 15 and demonstrated to be a cobamide-containing enzyme (Foster, Jones & Woods, 1961). The purified enzyme has now been shown to promote methionine synthesis by ultrasonic extracts of strain 121/176 grown on methionine, and to do so more efficiently than cobalamin.

Such extracts of strain 121/176 can also be activated, when EHC is cofactor, by small amounts of extracts of strain PA 15 or of *E. coli* 3/62 (a methionine auxotroph) grown on methionine in the absence of added cobalamin. This effect cannot be due to the provision of small amounts of cobamide–enzyme since it is not obtained when tetrahydropteroylmonoglutamate is the folic acid cofactor. This activating material has been purified several-fold from strain 3/62. It has the properties of a protein and has been designated '121/176-lesion enzyme'; it contains no cobamide–enzyme when assayed according to Foster *et al.* (1961).

Ultrasonic extracts of strain 3/62 can be activated to synthesize methionine, with EHC as cofactor, by small amounts of extract of strain PA 15 or strain 121/176; with tetrahydropteroylmonoglutamate, activation occurs only when cobalamin is present. The activating material has been partially purified from strain 121/176; it has the properties of a protein and has been designated '3/62-lesion enzyme'.

These observations suggest that *E. coli* can methylate homocysteine by two mechanisms. The 'normal' mechanism operates, in the absence of added cobalamin, via the '3/62-lesion enzyme' and the '121/176-lesion enzyme'. It uses EHC or tetrahydropteroyltriglutamate, but is inhibited by tetrahydropteroylmonoglutamate. When cobalamin is available, a second mechanism can operate via the '3/62-lesion enzyme' and the cobamide–enzyme; this pathway uses EHC, tetrahydropteroyltriglutamate *or* tetrahydropteroylmonoglutamate as cofactor. The reactions catalysed in these pathways are being investigated.

Foster, M. A., Jones, K. M. & Woods, D. D. (1961). *Biochem. J.* (in the Press).
Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
Guest, J. R. & Woods, D. D. (1960). *Biochem. J.* **77**, 422.
Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.

The Regulation of Amino Sugar Metabolism in *Bacillus subtilis*

By Juliet S. Clarke and C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

The formation of glucosamine 6-phosphate from hexose 6-phosphate and glutamine is a key reaction in the biosynthesis of amino sugars. Glutamine–hexose 6-phosphate transamidase (Ghosh, Blumenthal, Davidson & Roseman, 1960) has now been demonstrated to be present in the non-particulate fraction of ultrasonic extracts of *Bacillus subtilis* NCTC 1379. The crude enzyme has K_m 2 mM for glucose 6-phosphate and K_m 0·7 μM-for glutamine. It is inhibited 50% by 10 μM-diazo-5-oxo-L-norleucine (donated by Parke, Davis and Co.). It thus resembles rather closely the transamidase of *Escherichia coli* studied by Ghosh *et al.* (1960). Ultrasonic extracts of *B. subtilis* also

contain very low amounts of the enzyme glucosamine 6-phosphate deaminase (Roseman, 1959; Strange & Dark, 1960).

When an amino sugar such as *N*-acetylglucosamine (0·1 %) was added to growing cultures of *B. subtilis*, the synthesis of glucosamine 6-phosphate by ultrasonic extracts was apparently abolished. At the same time the activity of glucosamine 6-phosphate deaminase increased from an initial rate of deamination of $< 2{\cdot}5\,\mu$m-moles/mg. of protein/min. to one of 19·3. By fractionation of the extract with protamine sulphate the synthetic enzyme, glutamine–hexose 6-phosphate transamidase, could be separated from the degradative one, glucosamine 6-phosphate deaminase. It was then found that the transamidase had been repressed from an initial rate of $29{\cdot}5\,\mu$m-moles of glucosamine 6-phosphate synthesized/mg. of protein/min. to one of 2·9. This is therefore an example of the control of a reaction by two concerted effects, namely repression of a synthetic enzyme and induction of a degradative one.

Other degradative enzymes which are induced when *N*-acetylglucosamine (0·1 %) is added to growing *B. subtilis* are *N*-acetylglucosamine deacetylase (Rogers, 1949) and *N*-acetylglucosamine 6-phosphate deacetylase (Roseman, 1959).

Ghosh, S., Blumenthal, H. J., Davidson, E. & Roseman, S. (1960). *J. biol. Chem.* **235**, 1265.
Rogers, H. J. (1949). *Biochem. J.* **45**, 87.
Roseman, S. (1959). *Annu. Rev. Biochem.* **28**, 545.
Strange, R. E. & Dark, F. A. (1960). *Nature, Lond.*, **188**, 741.

Roberts, Abelson, Cowie, Bolton & Britten (1955) showed that incorporation of [^{35}S]sulphate into the proteins of *E. coli* is decreased by the presence in the growth medium of glutathione and is abolished by the presence of cystine. When synthesis of PAPS by extracts of *E. coli* ATCC 9723 grown under these conditions was investigated, it was found that glutathione (26·7 mg./l.) had no effect but that cystine (26·7 mg./l.) completely repressed PAPS formation. This suggests that *E. coli*, which grows equally well on sulphate, glutathione or cystine, utilizes cystine, but not glutathione, in preference to sulphate. Experiments on the incorporation of [^{35}S]sulphate by growing *E. coli* support this view. Since a later stage in sulphate assimilation, namely the reduction of inorganic sulphite to sulphide, also appears to be repressed by cysteine (Mager, 1960), the mechanism of co-ordinate repression (Ames & Garry, 1959) may be operative here.

The situation with respect to *B. subtilis* was found to be slightly different in that both cysteine (26·7 mg./l.) and glutathione (26·7 mg./l.) caused repression of PAPS synthesis when present in the growth medium. Incorporation of [^{35}S]sulphate into the cell constituents of *B. subtilis* was likewise decreased by both cysteine and glutathione.

Ames, B. N. & Garry, B. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1453.
Gregory, J. D. & Robbins, P. W. (1960). *Annu. Rev. Biochem.* **29**, 347.
Mager, J. (1960). *Biochem. biophys. Acta*, **41**, 553.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Washington, D.C.: Carnegie Institution of Washington.

The Repression of Sulphate Activation in *Escherichia coli* and *Bacillus subtilis*

By C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

Many micro-organisms utilize inorganic sulphate as sole source of sulphur. The first stage in the reduction of sulphate appears to be its activation by ATP to form adenosine 5′-phosphosulphate. This is followed in most cases by reaction with a further molecule of ATP to yield 3′-phosphoadenosine 5′-phosphosulphate (PAPS) (Gregory & Robbins, 1960). Ultrasonic extracts of *Escherichia coli* ATCC 9723 and of *Bacillus subtilis* NCTC 1379 have now been shown to contain both enzymes necessary for the synthesis of PAPS. Enzymic activity was measured by formation of PAP^{35}S from [^{35}S]sulphate, ATP and Mg^{2+} at pH 8·0 and was found to reside in the fraction not sedimentable at 105 000*g* for 60 min.

The Deoxyribonucleases of Bacteriophage-Infected *Escherichia coli*

By A. B. Stone* and K. Burton. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford*)

Extracts prepared from *Escherichia coli* after infection with certain bacteriophages (T2, T4, T5 or T6) show a higher deoxyribonuclease activity than similar extracts prepared from the normal bacterium (Pardee & Williams, 1952; Kozloff, 1953; Kunkee & Pardee, 1956; Crawford, 1959). It has been confirmed that the deoxyribonuclease in such extracts is partially inhibited by the endogenous ribonucleic acid, but the present experiments give

* Medical Research Council Training Scholar.

no evidence that infection results in the destruction of some of this inhibitor, as had been proposed earlier to explain the increase in enzyme activity (Kozloff, 1953). The results obtained indicate that infection with T2, T5 or T6 increases the rate of synthesis of deoxyribonuclease. In its pH optimum and preference for heat-denatured deoxyribonucleic acid as substrate, the enzyme which is formed by the infected cell resembles the alkaline phosphodiesterase which has recently been isolated from uninfected *E. coli* (Lehman, 1960), but there is some indication that it is a separate enzyme.

Crawford, L. V. (1959). *Virology*, **7**, 359.
Kozloff, L. M. (1953). *Cold Spr. Harb. Sym. quant. Biol.* **18**, 209.
Kunkee, R. E. & Pardee, A. B. (1956). *Biochim. biophys. Acta*, **19**, 236.
Lehman, I. R. (1960). *J. biol. Chem.* **235**, 1479.
Pardee, A. B. & Williams, I. (1952). *Arch. Biochem. Biophys.* **40**, 222.

The Activation of Streptococcal Deoxyribonuclease by Bivalent Cations

By A. B. Stone* and K. Burton. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford*)

The action of Streptodornase (Lederle Laboratories, N.Y., U.S.A.) required Mg^{2+}, Mn^{2+} or Fe^{2+}. This enzyme is a deoxyribonuclease prepared from the culture medium of group C streptococci. For Mg^{2+}, the optimum concentration was about 0·6 mM. Higher metal concentrations inhibited; for example, with 2·5 mM-Mg^{2+} the activity was about 15% of the maximum. Ca^{2+} or Sr^{2+} could not fulfil this requirement, but they increased the rate of reaction in the presence of Mg^{2+}, Mn^{2+} or Fe^{2+}. Even with no added Ca^{2+}, the reaction mixture contained 0·1 mM-Ca which was contributed by the DNA preparation.

The presence of sufficient Ca^{2+} prevented the inhibition by excess Mg^{2+}. With Mg^{2+} at its optimum concentration of 0·6 mM, estimation of the acid produced indicated that the reaction stopped when about 7% of the phosphodiester bonds of the DNA had been hydrolysed. When both Mg^{2+} and Ca^{2+} were present, each at 6 mM, the reaction was approximately 8 times faster, and about 17% of the bonds were cleaved. If the substrate was first heated at 100° for 30 min., it was not attacked unless a cation from each group was added.

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These results suggest that depolymerization of DNA by streptodornase involves two metal-binding sites, or groups of sites, one having a specific requirement for Mg^{2+}, Mn^{2+} or Fe^{2+} and the other for Ca^{2+} or Sr^{2+}.

Nicotinamide–Adenine Nucleotide-Linked Dehydrogenases of *Zymomonas mobilis* (*Pseudomonas lindneri*)

By D. W. Ribbons and E. A. Dawes. (*Department of Biochemistry, University of Glasgow*)

Zymomonas mobilis ferments glucose almost quantitatively to ethanol and carbon dioxide (Kluyver & Hoppenbrouwers, 1931); small amounts of lactate are also formed. With specifically labelled [^{14}C]glucose, Gibbs & DeMoss (1951, 1954) showed that the carbon dioxide is derived equally from C-1 and C-4 of the glucose molecule. Fermentation of [3:4-$^{14}C_2$]glucose by washed cell suspensions produced ethanol labelled in the methyl carbon atom, with only a trace of ^{14}C in the carbinol carbon atom. From these results, which are consistent with the operation of the Entner–Doudoroff (1952) pathway of glucose dissimilation, it has become generally assumed that *Z. mobilis* ferments glucose anaerobically by this hexose monophosphate pathway although, somewhat surprisingly, this assumption has never been tested at the enzymic level.

It was imperative therefore to demonstrate enzymically (*a*) the intermediary steps employed in the dissimilation of glucose, and (*b*) the nicotinamide–nucleotide specificity of the dehydrogenases concerned, so that a mechanism for the reoxidation of reduced coenzymes could be formulated in this microbe.

Sonic extracts of glucose–peptone-grown cells of *Z. mobilis* catalyse the formation of pyruvate from 6-phosphogluconate or 3-phosphoglycerate when hydrazine is added. Pyruvate accumulation could not be detected in the absence of hydrazine owing to an α-carboxylase [previously described by DeMoss (1953)] that catalyses acetaldehyde formation. Initial attempts to demonstrate NAD-linked dehydrogenases were hindered by the presence in crude extracts of a NADH oxidase. Ultracentrifugation of these extracts (105 000*g* for 2 hr.) sedimented the NADH oxidase activity and spectrophotometric assays of the supernatant fraction revealed the presence of dehydrogenases for glucose 6-phosphate, triose phosphate and ethanol. Triose phosphate and ethanol dehydrogenases are specific for NAD, but glucose 6-phosphate dehydrogenase can utilize either NAD or NADP as electron acceptor.

Glucokinase and gluconokinase were also demonstrated in the supernatant fraction by reduction of DPN when incubated with substrate and ATP. Crude extracts with added ATP converted both glucose and gluconate into pyruvate.

The results show that the enzymes required for an anaerobic Entner–Doudoroff pathway are present in extracts of *Z. mobilis* and indicate that the oxidation–reduction balance is probably maintained by reduction of DPN rather than TPN during glucose 6-phosphate oxidation, since transhydrogenase activity could not be detected even in crude extracts.

DeMoss, R. D. (1953). *J. cell. comp. Physiol.* **41**, Suppl. 1, 207.
Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
Gibbs, M. & DeMoss, R. D. (1951). *Arch. Biochem. Biophys.* **34**, 478.
Gibbs, M. & DeMoss, R. D. (1954). *J. biol. Chem.* **207**, 689.
Kluyver, A. J. & Hoppenbrouwers, W. J. (1931). *Arch. Mikrobiol.* **2**, 245.

An Iron-Containing Growth Factor from *Mycobacterium tuberculosis*

By G. A. Snow. (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

Extracts of various mycobacteria promote the growth of *Mycobacterium johnei* on laboratory media (Twort & Ingraham, 1913). Mycobactin, the growth factor from *M. phlei*, was isolated (Francis, Macturk, Madinaveitia & Snow, 1953) and its structure examined (Snow, 1954*a*, *b*). A growth factor from *M. tuberculosis* (strain H 37 av.) has now been concentrated. This organism, like *M. phlei*, only produces substantial amounts of growth factor when it is grown on glycerol–beef infusion broth; growth is rather poor on this medium.

The moist, washed, mycobacteria (5 kg.) were treated with methanol to give a dry powder (940 g.) which was extracted with cold ether. The extract was concentrated, impurities were precipitated with acetone, and the filtrate was evaporated. The residue (58·5 g.) was partitioned between light petroleum and aqueous ethanol. Evaporation of the lower layer left a brown wax (6·5 g.) which was purified by chromatography in benzene and chloroform on alumina; the product (0·77 g.) had about 15% of the growth-promoting activity of mycobactin towards *M. johnei*. It was a deep reddish brown wax with an absorption spectrum having a maximum at 445 mμ ($E^{1\%}_{1\,cm.}$ 6·2) resembling that of crystalline ferric mycobactin ($E^{1\%}_{1\,cm.}$ 41·0 at 445 mμ). Further chromatographic purification has yielded material with an extinction coefficient 80–90% that of ferric mycobactin, but so far no crystalline product has been obtained. A chloroform solution of purified material was shaken with 5N-HCl; the colour was discharged and iron passed into the acid. The absorption spectrum of the metal-free material resembled that of mycobactin, but with the peaks shifted to lower wavelengths: maxima (mycobactin in brackets) 241 (250), 249 (258, shoulder), 304 (311) mμ. The spectrum corresponded closely to that of an equimolar mixture of 2-*o*-hydroxyphenyloxazoline and *N*-ethyloctadec-2-enohydroxamic acid [cf. structural considerations for mycobactin (Snow, 1954*b*)]. The growth factor from *M. tuberculosis* thus resembles mycobactin but has a somewhat different structure. Its solubility and chromatographic properties are also distinct. Under suitable conditions in liquid medium both mycobactin and the tubercle growth factor produce optimum growth of *M. johnei* at 30 μmg./ml., and detectable stimulation at 4 μmg./ml.

The natural occurrence of the factor as an iron complex is of interest in relation to the iron-containing growth factors which have been found in other micro-organisms (cf. Bickel *et al.* 1960).

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The Heterogeneity of Crystalline Yeast Alcohol Dehydrogenase as shown by Starch-Gel Electrophoresis

By D. C. Watts,* C. Donninger and E. P. Whitehead. (*Department of Biochemistry, University College London*)

Crystalline yeast alcohol dehydrogenase, prepared by the method of Racker (1950), could be resolved into two components in the Spinco model E analytical ultracentrifuge at pH 7·5 (0·05M-phosphate buffer) and 2° with a rotor speed of 59 780 rev./min. A symmetrical peak separated from a slower, small asymmetric peak in a 4-month-old preparation (stored at −15°). Hayes & Velick (1954) and Racker (1950) have shown by electrophoresis the presence of two components, the proportions of which varied according to the age of the sample.

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Alcohol dehydrogenase, when investigated by starch-gel electrophoresis, showed little movement from the origin at pH 7·5 (0·05M-phosphate buffer), but at pH 9·0, using the discontinuous buffer system of Poulik (1957), could be separated into 18 components as revealed by staining with naphthalene black. Five bands showed enzyme activity when the gel was treated with a mixture of substrates, 2:4-dichlorophenolindophenol and *N*-methylphenazonium methosulphate. Preparations using fresh yeast samples gave consistently the same pattern, as did a sample obtained from Boehringer Ltd. Recrystallization was without effect on the electrophoretic pattern. Dialysis of the enzyme with chelating agents suggested that one, at least, of the active bands was a complex between metal ion, phosphate (used for the preliminary dialysis) and the enzyme. When care was taken to free buffers and starch from metal ions, the effect of chelating agents on the enzyme almost disappeared. No effect compatible with the formation of subunits, as observed by Kagi & Vallee (1960), could be detected by starch-gel electrophoresis on treating the enzyme with 1:10-phenanthroline.

We thank Mr D. B. Gammack for carrying out the ultracentrifuge experiment. One of us (E.P.W.) is indebted to the Medical Research Council for a personal grant.

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Acidic Mucosubstances of Cortical Bone

By G. M. Herring and P. W. Kent. (*Medical Research Council, Bone-Seeking Isotopes Research Unit, The Churchill Hospital, Oxford and the Department of Biochemistry, University of Oxford*)

Treatment of bovine cortical bone powder with a solution of sodium salts of ethylenediaminetetraacetic acid (EDTA) (about 40%, pH 7·9) for three successive periods of 48 hr. brings about removal of 6·6% of total organic constituents as well as inorganic salts (cf. Dische, Danilczenko & Zelmenis, 1958). The residual matrix contains considerable amounts of bound carbohydrate which has been extensively studied in the past (Rogers, 1951; Glegg & Eidinger, 1954; Meyer, Davidson, Linker & Hoffman, 1956; Dische *et al.* 1958).

Free electrophoresis at pH 8 in 0·1M-diethyl barbiturate buffer indicated the presence of at least four substances soluble in EDTA. One electrophoretically separated component was a form of soluble collagen (12·9% hydroxyproline, 1·3% hexose, 0·5% amino sugar). This, with non-acidic proteins, could be removed on Amberlite CG-50 resin in 0·2M-sodium phosphate buffer (pH 5·2) (Partridge & Davis, 1957).

Two acidic mucosubstances, staining with toluidine blue, were obtained from the collagen-free extract by fractionation with cetylpyridinium chloride in the presence of Celite (Korn, 1959) and extraction of the resulting precipitate with 0·3M- and 1·2M-sodium chloride solutions (Schiller & Dorman, 1960). The first acidic constituent, which readily oxidized under the conditions of the periodate–Schiff reaction, contained (K salt) 6·4% amino sugar, 0·7% uronic acid, 13·6% hexose, 2·4% methylpentose, 15·9% neuraminic acid, the hexose/amino sugar ratio being 2:1 and the neuraminic acid/amino sugar ratio, 7:5 [cf. orosomucoid (Winzler, 1958)]. The remainder of the substance appeared to be polypeptide.

The second acidic constituent, identified as chondroitin sulphate, was metachromatic and did not react in the periodic acid–Schiff test.

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Erythritol in Bovine Foetal Tissue

By A. E. Williams, J. H. Pearce and H. Smith. (*Microbiological Research Establishment, Porton, Wilts.*)

During an investigation of the chemical basis for the preferential growth of *Brucella abortus* in bovine foetal tissue (Smith *et al.* 1961) a material, which stimulated the growth of *Br. abortus*, was isolated from allantoic fluid and identified as erythritol.

The diffusate (16·5 g.) from bovine allantoic fluid (536 ml.) was applied to a mixed-bed resin column (Amberlite IR-45 and Zeo-Karb 225). The water eluate (5·1 g.) contained several reducing sugars which were removed by passage through a highly basic resin [Amberlite IRA-400 (OH)]. The freeze-dried water eluate (0·34 g.) was chromato-

graphed on paper (Whatman no. 3MM) in butanol–pyridine–water (6:4:3, by vol.). Material stained by the method of Trevelyan, Procter & Harrison (1950) and having $R_{glucose}$ 1·4 was eluted with water, freeze-dried and crystallized from acetone containing a trace of water. The substance (105 mg.) had C, 39·1; H, 8·7; calc. for $C_4H_{10}O_4$: C, 39·3; H, 8·3%. The following criteria showed it to be erythritol: m.p. and mixed m.p. with authentic erythritol, 119–120°; infrared spectrum; paper chromatography in butanol–pyridine–water (6:4:3, by vol.), in butanol–acetic acid–water (4:1:5, by vol.) and in ethyl acetate–pyridine–water (12:5:4, by vol.); apparent (associated) mol.wt. by Rast's method (386; authentic erythritol had mol.wt. 398 examined at same concentrations); the dibenzylidene derivative had m.p. 198–199° and mixed m.p. 197–198° with an authentic sample. Finally authentic erythritol was equally effective in stimulating the growth of *Br. abortus*.

A survey of bovine maternal and foetal tissues showed that erythritol was only a foetal product and was concentrated in the chorion, the allantoic fluid and the placental tissue.

Until recently erythritol had not been found in animals and was regarded as metabolically inert (Carr & Krantz, 1945; Batt, Dickens & Williamson, 1960). However, while this work was in progress erythritol was found in human urine (Touster, Hecht & Todd, 1960). The questions therefore arise as to how erythritol is produced and what is its function. It may act as an intermediate between erythrose and erythrulose as sorbitol acts between glucose and fructose in the foetal tissues of ungulates (Hers, 1958).

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Hydrolytic Enzymes of *Phaseolus vulgaris* acting on the Raffinose Family of Oligosaccharides (including Sucrose)

By R. A. Cooper and R. N. Greenshields. (*Department of Biochemistry, University of Birmingham*)

The soluble reserve carbohydrates of the French bean *Phaseolus vulgaris*, L., namely, verbascose, stachyose, raffinose and sucrose, occur in both the embryo and cotyledons of the ungerminated seed (French, 1954; Duperon, 1955; Greenshields, 1960). On germination, the galactose oligosaccharides disappear leaving mainly sucrose in the cotyledons and sucrose, glucose and fructose in the embryo. No free galactose was found at any stage of germination (Greenshields, 1960).

A survey was made of the hydrolytic enzymes in the bean which act on these saccharides. The cotyledons of steeped and germinated beans possessed an α-galactosidase activity (optimum pH 5·0) and a sucrase activity ('alkaline sucrase', optimum pH 7·7). Embryos of steeped seeds possessed similar enzyme activities but after germination for 3 days another sucrase (optimum pH 4·9) was present besides the 'alkaline sucrase' and the α-galactosidase. At later stages of germination the 'alkaline sucrase' tended to disappear.

Some properties of these enzymes have been studied using crude and partially purified extracts. The action of the α-galactosidase was typical of other plant α-galactosidases (Courtois, Petek & Anagnostopoulos, 1954). The sucrase, optimum pH 4·9 (which has been purified some 80 times), appeared to be a β-fructosidase, while the 'alkaline sucrase' appeared to be specific for sucrose.

Regarding the function of these hydrolytic enzymes *in vivo*, it was noted that the parts of the plant without the β-fructosidase lacked free reducing sugars although sucrose was present. The appearance of this enzyme in the embryos on germination coincided with the appearance of glucose and fructose. In contrast, the presence of 'alkaline sucrase' was not associated with free reducing sugars although sucrose was always present. The function of the α-galactosidase is not clear. It was present before and after the galactose oligosaccharides were utilized by the plant and yet no free galactose appeared. Similar results were found when stachyose and raffinose were absorbed by dissected plants at later stages of germination, when the α-galactosidase was still present but when the saccharides present before germination had disappeared. The stachyose and raffinose were rapidly metabolized but again no free galactose was detected. This indicates that *in vivo* the α-galactosidase is inoperative or that the galactose liberated by its action is rapidly metabolized and not allowed to form a pool.

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A Contribution to the Chemical Structure of the Cell Wall of *Micrococcus lysodeikticus*

By J. W. Czerkawski, H. R. Perkins and H. J. Rogers. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The cell-wall material from *Micrococcus lysodeikticus*, unlike that from some other micro-organisms, consists almost exclusively of mucopeptide components. It is, therefore, particularly well suited for detailed chemical study. A structure has been suggested (Brumfitt, Wardlaw & Park, 1958) which is based on that of the small uridine mucopeptide isolated from penicillin-treated staphylococci (Park, 1952; Park & Strominger, 1957) as far as the attachment of the amino acids is concerned, together with the concept of a polysaccharide chain as a backbone. Other workers (Perkins & Rogers, 1959; Perkins, 1960; Salton & Ghuysen, 1960; Ghuysen, 1961) have isolated a variety of fragments as a result of either dilute acid or enzymic hydrolysis. However, it is still unprofitable to attempt to construct a probable total structure for the mucopeptide or mucopeptides in the wall. The present paper is concerned with a partial attempt to assess the likelihood that the suggested structure is correct and the importance for the total structure or structures of the various fractions which have been isolated, often in very small yield.

The number of polar groups in both the whole insoluble mucopeptide and in lysozyme digests of this material has been determined. This has been done both by direct titration and by dye-binding techniques. Both the insoluble mucopeptide and the soluble enzymic digests showed the presence of the same number of polar groups. The number of free carboxyl groups was approximately that to be expected from the Brumfitt *et al.* (1958) type of structure. The small shift in the titration curve for the soluble material as compared with that for insoluble mucopeptide was consistent with the rupture of hydrogen bonds. Only about half the ϵ-NH_2 groups of lysine could be titrated.

Estimation of the content of the amino sugars (glucosamine and muramic acid) by a variety of available techniques after different periods of acid hydrolysis has allowed an accurate correction for the slight destruction during the process of liberation from the mucopeptide. When ammonia arising from this source has been allowed for, about half a mole of extra ammonia per mole of glutamate residue is seen to be liberated by short hydrolysis with acid. The titration results would make it unlikely that this was originally present in the form of amide groups. Examination of the acetyl content of the mucopeptide material has shown that there is again about half a mole of acetyl per mole of glutamate over and above that necessary for acetylation of the amino groups of the sugars. The volatile acid liberated by acid hydrolysis was shown to be acetic acid by paper chromatography. *O*-Acetyl groups were not present.

Taken together the results suggest that the Brumfitt *et al.* (1958) type of structure may be correct in part, but that a considerable number of the ϵ-NH_2 groups of lysine are linked in such a way that they are no longer functional; the excess acetyl content may be involved here. There is, however, within the molecule a linkage or compound which on brief acid hydrolysis gives rise to ammonia. The nature of any such linkage or compound is, of course, at present unknown.

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Polysaccharides of *Chlorella pyrenoidosa*

By S. A. Olaitan and D. H. Northcote. (*Department of Biochemistry, University of Cambridge*)

Three polysaccharides, hemicellulose *A*, $[\alpha]_D$ + 11°, hemicellulose *B*, $[\alpha]_D$ + 75° and *Chlorella* starch, $[\alpha]_D$ + 135°, present in the ratio 9:2:1, have been obtained electrophoretically pure from the alkali-soluble polysaccharides of *Chlorella*. Three purification procedures were used, namely, (*a*) treatment with salivary amylase, (*b*) separation on a glass-powder electrophoresis column (Hocevar & Northcote, 1957) and (*c*) fractionation with cetyltrimethylammonium bromide.

The molecular weights of hemicelluloses *A* and *B* calculated from $S_{20,w}$ and $D_{20,w}$ were 32 000 and 18 000 respectively. The molecules were asymmetrical with axial ratios for *A* of 15 and *B* of 30 for prolate ellipsoids.

Hemicellulose *A* contained galactose (7·0 moles), rhamnose (5·0 moles), arabinose (3·0 moles), xylose (3·0 moles), mannose (2·0 moles) and glucose (1·0 moles). Enzymic, methylation and periodate studies have shown that the polysaccharide is a branched β-linked galactorhamnan. Galactose and mannose monomers function as terminal non-reducing ends and branching occurs on galactose residues. A crystalline trisaccharide containing galactose, arabinose and rhamnose has been obtained by acetolysis. Further proof of the homo-

geneity of the material was obtained by labelling the reducing end with sodium [^{14}C]cyanide, and calculating the number-average molecular weight as 35 000 (Moyer & Isbell, 1958).

Hemicellulose *B* contained galactose (13·0 moles), glucose (25·0 moles), mannose (2·0 moles), arabinose (1·0 moles), xylose (1·0 moles), rhamnose (12·0 moles), and an unknown sugar (20·0 moles). A benzoylhydrazone of the unknown sugar had m.p. 146°. Periodate oxidation indicated that the polysaccharide was branched.

Chlorella starch, which was closely associated with hemicellulose *B*, contained glucose units only and periodate oxidation showed that it had an apparent chain length of 9 units. It was completely degraded by salivary amylase only when separated from hemicellulose *B*. It contained 7% of amylose.

The structures of these polysaccharides have been related to their function and organization in the cell wall as seen with the electron microscope.

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A Mucopolysaccharide from Human Aorta

By Helen Muir.* (*Medical Unit, St Mary's Hospital, London, W.* 2)

The wall of the aorta contains several sulphated mucopolysaccharides (Buddecke, 1960; Kaplan & Meyer, 1960; Meyer, Davidson, Linker & Hoffman, 1956) including heparitin or heparin monosulphate (Jorpes & Gardell, 1948) and the chondroitin sulphates which constitute the main fraction. In addition to these a component of low sulphate content has been isolated.

Acetone-dried aortas were rehydrated and digested with pancreatin at pH 7·2 and the mixed mucopolysaccharides purified from protein (Muir, 1958). During this procedure the pH values of the solutions varied between 5·8 and 7·2. Preparative electrophoresis at pH 7 in phosphate buffer on glass fibre that was kept under an external pressure to prevent imbibition, gave a component which separated clearly from the bulk of the mucopolysaccharides. The course of the electrophoresis could be followed by taking prints of the surface of the block by pressing on to it dry filter paper strips which were then stained with toluidine blue. The appropriate area was cut out and the glass fibre removed by filtration. The fibre was washed and the filtrates concentrated and desalted with Sephadex G-25 (Pharmacia, Uppsala, Sweden). The mucopolysaccharide was precipitated with ethanol containing sodium acetate. After repeated electrophoresis the material ran as a single spot during cellulose acetate electrophoresis at pH 7.

Although the mucopolysaccharide contains glucosamine (Gardell, 1953; Crumpton, 1959) and is resistant to hyaluronidase its analysis differs from that of heparin monosulphate from liver (Jorpes & Gardell, 1948) and chick oviduct (Schiller, 1959) and from by-products of commercial heparin (Cifonelli & Dorfman, 1959). Its infrared spectrum also differs from that of the heparin monosulphate from chick oviduct (Schiller, 1959). The cetylpyridinium salt (Scott, 1955) is eluted from a cellulose column at 26° (Buddecke, 1960) by 0·7 M-KCl. The mucopolysaccharide had $[\alpha]_D + 78°$ and contained the following constituents (molar ratios): glucosamine (1·0); uronic acid (carbazole) (1·4); sulphate (0·58); acetyl (1·01); sodium (1·47).

The mucopolysaccharide, which had no antithrombin or fat-clearing activity, was found both in the intima and media of the aorta, but no fraction having a similar mobility during cellulose acetate electrophoresis was detected in mucopolysaccharide mixtures from liver, spleen, subcutaneous tissue, cartilage or urines from six patients with Hurler's syndrome.

* Pearl Research Fellow.

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Some Evidence for a Coenzyme-linked Dehydrogenase System in the Brain

By J. W. Ridge. (*Burden Neurological Institute, Stapleton, Bristol*, 5)

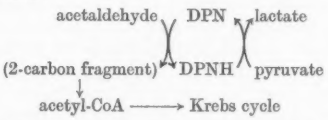
It has recently been shown (Ridge, 1961) that following the administration of ethanol to the rat (3 ml., 30% by stomach tube) the brain lactate concentration went into a series of slow oscillations that lasted about an hour before returning to the normal level. This observation was of interest in

view of the apparent independence of the brain lactate on conditions outside the brain. The lactate changes were therefore thought to reflect an involvement of ethanol (or one of its metabolites) in brain carbohydrate metabolism.

To investigate this connexion further the pyruvate and α-oxoglutarate concentrations were estimated in the rat brain following the administration of ethanol. The method of Koepsell & Sharpe (1952) was used and the rats were killed by immersion in liquid oxygen.

Pyruvate and α-oxoglutarate changes were found and they were absolutely synchronous with the lactate changes. The oscillations of α-oxoglutarate concentration were in phase with the lactate changes (i.e. maxima and minima occurred in both at the same time after ethanol administration) but the pyruvate changes were 180° out of phase with the lactate changes (i.e. a maximum in lactate concentration occurred at the same time as a minimum in the pyruvate concentration and vice versa).

These results suggested that the following system was operating in the brain during ethanol metabolism:



Since the brain appears to have only a limited ability to oxidize ethanol to acetaldehyde it was assumed that the bulk of the acetaldehyde—which is known to be capable of acting as a substrate in brain metabolism—arose from the oxidation of ethanol in the liver.

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Respiratory Control in Brain Mitochondrial Preparations

By D. Biesold* and R. Balázs. (*Medical Research Council, Neuropsychiatric Research Unit, Woodmansterne Road, Carshalton, Surrey*)

The stimulation of the respiration of rat-brain mitochondrial preparations by 0·05 mM-dinitrophenol (DNP) was studied under the conditions and using the standard medium previously described (Balázs, 1959). The stimulation depended on the substrate used (all substrates were used at 10 mM-concentration). With pyruvate alone, DNP increased the respiration 3–4 times. With α-oxoglutarate, or α-oxoglutarate and malonate, DNP had little effect. With succinate, DNP inhibited the respiration. When the substrates α-oxoglutarate or succinate were combined with 10 mM-pyruvate, or when pyruvate was supplemented with 2 mM-malate, the respiratory rate was relatively high and DNP increased the respiration 2–3 times.

The stimulation of respiration with 2 mM-ADP also depended on the substrate. With α-oxoglutarate the respiration was increased 4 times: with pyruvate it was doubled. With succinate the main effect of ADP was to prevent the falling off of respiration during incubation. When the substrates were combined as before, the respiration was doubled in every case.

In the presence of added ADP, DNP produced a further stimulation (200%) with pyruvate as substrate; with α-oxoglutarate it had little effect and with succinate it inhibited respiration. With the combined substrates DNP increased the respiration by only 20–50%. Since brain mitochondrial preparations contain Mg-dependent ATPase which is inhibited by fluoride, these experiments were repeated in the presence of 10 mM-NaF; under these conditions with pyruvate and malate as substrates, the respiratory control ratio (Chance, 1959) was about 2. DNP then stimulated respiration with combined substrates 2–3 times.

Yeast hexokinase (9 units) and glucose in the presence of ADP and NaF produced the same stimulation of respiration as DNP, but no stimulation was observed in the absence of added ADP. In liver mitochondrial preparations under similar conditions, hexokinase doubled the rate of respiration.

When inorganic phosphate was made rate-limiting in a system containing hexokinase, glucose and fluoride, as well as combined substrates, addition of phosphate increased the respiration rate 3 times.

Similar results were obtained with mitochondrial preparations obtained with Dounce, Aldridge or Nird homogenizers, and using 0·25M- to 0·4M-sucrose solutions. The preparations were stable in the resting state and gave almost linear respiration for up to 60 min. at 37·5°. Stimulation of respiration by DNP or inorganic phosphate was observed in preparations pre-incubated for 30–40 min.

It is concluded that respiratory control can be obtained in brain mitochondrial preparations to an extent depending on the experimental conditions.

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The Localization and Action of Cerebral Phosphoprotein Phosphatase

By S. P. R. Rose. [*Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E.* 5]

The partial purification and properties of a cerebral phosphoprotein phosphatase have been described (Rose & Heald, 1960, 1961). The enzyme was labile, activated by Mg^{2+} and highly specific. This report describes the localization of the enzyme and discusses its mode of action.

Sections of fresh ox brain, dissected out and dispersed with water, showed activity varying from 9 μ moles of α-casein P/g. wet wt./hr. for corpus callosum to 23 μ moles of α-casein P/g. wet wt./hr. for parietal cortex in the absence of added activators. Activity was high in the caudate and cerebellar cortex, low in the medulla and floor of the fourth ventricle and intermediate in the thalamus, putamen and globus pallidus. This distribution can be contrasted with that found for other cerebral phosphatases (Gordon, 1953).

With guinea-pig cerebral cortex, dispersed in 0·32 M-sucrose containing mM-$MgCl_2$ pH 6·8, differential centrifugation revealed a distribution for the enzyme of 30–33% in the nuclear and heavy mitochondrial fractions and 60–65% in the soluble supernatant. Negligible quantities were found in the light mitochondria and microsomes. These proportions could not be altered by varying the extraction medium. Distribution between nuclear and mitochondrial fractions, however, depended on the centrifugal speeds at which the fractions were collected. Density-gradient centrifugation of the nuclear fraction suggested that the enzyme occurred as a mitochondrial contaminant of the nuclei. Within the mitochondrial fraction phosphoprotein phosphatase and succinic dehydrogenase activities could not be separated. This distribution resembles that found for phosphoprotein (Heald, 1959), fractions high in phosphatase also being rich in phosphoprotein as determined by release of inorganic phosphate on alkaline hydrolysis of acid-precipitated protein (A. Schwartz & R. Rodnight, unpublished results). It is considered that this is a further indication of the specific relationship of the enzyme and its endogenous phosphoprotein substrate.

This relationship has been further explored with purified enzyme preparations. Experiments with ^{32}P and enzymically dephosphorylated phosvitin failed to show incorporation of radioactivity in the presence of the enzyme, nor has it been possible to demonstrate catalysis of an ATP $\rightleftharpoons$ phosphoprotein transfer reaction by the phosphatase. These findings cast some light on the role played by the phosphatase in cerebral phosphoprotein metabolism.

The advice of Dr H. Hillman and Dr R. Rodnight, and a scholarship from the Medical Research Council, are gratefully acknowledged.

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The Metabolism of Elaeostearic Acid in the Rat

By T. Moore and I. M. Sharman. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Cambridge*)

α-Elaeostearic acid, the main fatty acid of tung oil, has three conjugated double bonds, which cause intense absorption at 272 mμ. It is already known that after ingestion by the rat or chicken elaeostearic acid is largely converted into an acid absorbing at 232 mμ, indicative of only two conjugated double bonds (Miller, Brown & Burr, 1938; Cruickshank, Houston & Moore, 1939). Young rats given a diet containing 30% of tung oil continued to grow, and remained in fair health, for many months. When they were killed their adipose tissues contained nearly 50% of the acid absorbing at 232 mμ. Extracts of their organs, including the intestinal walls but with the exception of brain, all showed intense absorption at 232 mμ. In contrast, extracts of the contents of the intestinal tract still absorbed at 272 mμ, which suggests that conversion took place in the intestinal walls. Estimation of the mean molecular weight of the mixed acids from the fat of a rat fed upon tung oil, and their analysis by gas chromatography, indicated that the acid absorbing at 232 mμ had the same length of carbon chain (C_{18}) as the elaeostearic acid from which it was formed. Hydrogenation of one of the double bonds of elaeostearic acid seems the most probable cause of its conversion into the acid absorbing at 232 mμ.

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The Identification of Lifschütz-Positive Material Appearing in the Cholesterol Fraction of Lipid Extracts

By G. S. Boyd and E. Barbara Mawer. (*Department of Biochemistry, University of Edinburgh*)

Lipid extracts, made under strictly anaerobic conditions, from a variety of mammalian organs have been subjected to silicic acid chromatography using light petroleum (b.p. 60–80°) containing increasing concentrations of $CHCl_3$. The fractions eluted with 50% (v/v) $CHCl_3$ in light petroleum contained all the unesterified cholesterol, but on paper chromatography in a reversed-phase system [using paper impregnated with liquid paraffin and $CHCl_3$–MeOH–paraffin (4:5:1, by vol.)], the fractions were shown to contain small amounts of a second compound which stained blue with ethanolic phosphotungstic acid (Martin, 1957). Cholesterol stains pink with this reagent. In some extracts traces of other compounds of low mobility were visible in ultraviolet light.

The second component has been separated from cholesterol on a reversed-phase column (Danielsson, 1958). The material gave a turquoise-blue colour with Lifschütz reagent, prepared according to Bergström & Wintersteiner (1941). This reaction is characteristic of 7-hydroxycholesterol, but since the chromatographic behaviour of the material was consistent with that of a monohydroxy compound, it seemed likely that it was an ester of 7-hydroxycholesterol. Infrared analysis confirmed the presence of an ester group at the C-3, and on mild alkaline hydrolysis material was obtained which was chromatographically identical with 7α-hydroxycholesterol.

A series of 3β-acyl esters of 7α-hydroxycholesterol (lauryl to stearyl) was synthesized using a modification of the method described by Henbest & Jones (1948) for the acetyl ester. These esters were labile and dehydration occurred on alumina columns giving cholestadienes and trienes, while in the presence of ether or on exposure to ultraviolet light, Lifschütz-negative products were formed.

The synthetic esters were chromatographically identical with the isolated material in the three systems described.

The naturally occurring material, which was found in extracts of human and rat serum and in rat skin and liver, may have arisen from cholesterol esters by an oxidation or peroxidation reaction at C-7 (Bergström, 1942).

Experiments with pancreatin powder have shown that identical material can also be produced enzymically by esterification of 7α-hydroxycholesterol with the appropriate fatty acids.

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Formalin Extraction of Lipids from Rat-Liver Nuclei

By P. B. Gahan. (*Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2*)

It was suggested, from histochemical observations, that during formalin fixation of tissues lipid material was lost from the heterochromatic regions of interphase nuclei (Chayen, Gahan & La Cour, 1959). That formalin could extract lipid material was confirmed by the demonstration of fatty acids and glycerophosphate (Chayen, Chayen & Aves, 1960). Since this work was concerned with whole tissues, it seemed desirable to re-investigate the effect of formalin on isolated nuclei.

Nuclei were isolated by homogenization in sucrose–citrate (Frazer & Davidson, 1953) and treated with 1% $CaCl_2$ in 4% formaldehyde (Baker, 1946). On shaking the fixative with $CHCl_3$ solid matter was obtained which stained positively for phospholipids (the acid haematein reaction and the Sudan black B stain); and for polysaccharide material [suitably controlled periodic acid–Schiff reaction (Hotchkiss, 1948)].

To confirm these results, the material was hydrolysed in boiling 0·5N-KOH in 95% ethanol for 2 hr. and examined by paper chromatography. Stearic and palmitic acids, glycerophosphate and choline were indicated. Further hydrolysis of the KOH hydrolysate for 3 hr. in 6N-HCl yielded a number of ninhydrin-positive spots. Much material remained at the starting line when 80% aqueous propanol was used as solvent.

Another sample was hydrolysed with saturated aqueous $Ba(OH)_2$ for 7 hr. Paper chromatography of a portion (80% propanol solvent) showed that the material remained at the starting line. The remainder was refluxed for 1¾ hr. in conc. HCl (McKibbin & Taylor, 1949). A spot having R_F 0·19 in 80% propanol and R_F 0·93 in ethanol–acetic acid–water (10:1:12, by vol., saturated with phenol) gave positive reactions for reducing sugars (Trevelyan, Procter & Harrison, 1950), for phosphate (Burrows, Grylls & Harrison, 1952) and for choline (Levene & Chargaff, 1950); no material remained at the starting line.

Thus the demonstration of palmitic and stearic acids, glycerophosphate and choline, has confirmed that nuclei do contain lipid matter which is ex-

tractable by formalin. From the mode of isolation, the periodic acid–Schiff reaction and the hydrolysis data, this appears to be a rather complex lipid which may contain polysaccharide material.

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Inhibition of 'Conjugate' Formation by Ethanol and Propylene Glycol during Metabolism of [4-^{14}C]Progesterone by Female-Rat Liver *in vitro*

By B. A. Cooke and W. Taylor. (*Department of Physiology, The Medical School, King's College, University of Durham, Newcastle upon Tyne* 1)

When [4-^{14}C]progesterone, dissolved in aqueous ethanol, was incubated in air with female-rat liver homogenate at a tissue:steroid ratio of approx. 56 000:1 a variable proportion (0–58%) of the radioactivity was not extractable from aqueous solution by hexane–chloroform at pH 7–8. After successive hydrolysis of the aqueous solutions with limpet-powder β-glucuronidase, cold and finally hot acid, more radioactivity was extractable from the neutralized solutions by hexane–chloroform, thus indicating that water-soluble 'conjugates' had been formed during metabolism (Cooke & Taylor, 1960). Further experiments showed that more consistent and extensive 'conjugation' of metabolites (60–90%) occurred when ethanol was omitted from the medium and incubations were carried out under oxygen.

Because of the extensive use of ethanol and propylene glycol for dissolving steroids before addition to incubation media in *in vitro* studies, the effect of these alcohols on 'conjugate' formation was investigated. Both ethanol and propylene glycol have been found to inhibit 'conjugation' of [4-^{14}C]progesterone metabolites. When 1·5 m-moles of ethanol (0·087 ml.) were added to the incubation medium (3 ml.) 'conjugate' formation was inhibited by 25–60% in air and 15–25% in oxygen; the corresponding values for propylene glycol (0·11 ml.) were 15–20% in air and 5–11% in oxygen.

In most experiments radioactivity released by limpet-powder β-glucuronidase decreased as the amount of ethanol or propylene glycol was increased. However, radioactivity released by cold acid hydrolysis usually increased. When ethanol or propylene glycol were present in the incubation media the total recovery of radioactivity after all types of hydrolysis was approx. 10% higher than in alcohol-free media. This suggests that the alcohols inhibit formation of water-soluble metabolites which may be 'conjugates' not rendered hexane–chloroform-soluble by the methods of hydrolysis used.

Ethanol and propylene glycol did not affect the amount of [4-^{14}C]progesterone metabolized, so it is possible that the alcohols inhibited steroid 'conjugate' formation by competing for endogenous conjugating systems, rather than for enzymes or cofactors involved in progesterone metabolism.

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The Evaluation of Radiochromatograms by Liquid Scintillation Counting and Photography

By S. R. Stitch and R. E. Oakey. (*Medical Research Council, Radiobiological Research Unit, Harwell, Didcot, Berks.*)

Many devices and techniques have been described for the evaluation of radioactive isotopes on paper chromatograms (Broda, 1960). Radioautography usually gives a qualitative image of activity only, and, with trace sources, may take many weeks or even months for development. Both windowless-flow and end-window counters have been adopted for this purpose, but with weak β-emitting isotopes, such as ^{14}C, the efficiency of the latter methods is low. Liquid scintillation counting may be used for the direct assay of radioactivity on paper chromatograms (e.g. Steenberg & Benson, 1956; Seliger & Agranoff, 1959; Wang & Jones, 1959; Geiger & Wright, 1960). Sources of error and variables in the use of this method have been pointed out by Loftfield & Eigner (1960).

We have used the liquid scintillation technique in conjunction with sensitive autopositive photography for partial characterization of ^{14}C-labelled

oestrogen metabolites by paper chromatography (Oakey, 1961) following isotope dilution. After chromatography of radioactive metabolites in admixture with carriers, a strip $1\frac{1}{4}$ in. wide is cut lengthwise from the chromatogram. This strip is then divided crosswise into $\frac{1}{2}$ in. segments, each of which is assayed for radioactivity by immersing directly in liquid scintillator (Stitch, 1959) and counting on the Ekco N612 liquid scintillation counter. The remainder of the chromatogram is sprayed with Turnbull's blue reagent (Barton, Evans & Gardner, 1952) to locate the carrier oestrogens, washed in water and dried. The chromatogram is placed directly under Kodak Autopositive photographic paper on the ground glass window of a specially constructed printing box. Suitable exposure of the photographic paper is given from 8×60w tungsten bulbs within the box. The exposed paper is then treated with Kodak Duostat developer, fixer, and washed in water. An intense black image of the chromatogram spot on a light background is recorded by the autopositive paper. The slow speed of the paper permits all photographic processing to be carried out in daylight.

By comparing the graphical record of the radioactivity with the photographic record of the carrier, useful and permanent evidence is obtained for the nature of the radioactive metabolites. The method should be of general application where paper chromatography is used to assist in the evaluation of radioactive isotopes by isotope dilution.

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The Separation of Steroid Oestrogens by Partition Chromatography

By R. E. Oakey (introduced by S. R. Stitch). (*Medical Research Council, Radiobiological Research Unit, Harwell, Didcot, Berks*)

Partition chromatography on paper (e.g. Axelrod, 1953; Mitchell & Davies, 1954) and on columns (e.g. Aitken & Preedy, 1956; Bauld, 1956; Givner, Bauld & Vagi, 1960) has been used extensively for the separation of steroid oestrogens. In the course of studies on the biosynthesis and metabolism of oestrogens we have devised solvent systems for the separation of certain epimeric oestrogens and of mixtures of widely differing polarity.

Paper chromatography in a light petroleum (b.p. 80–100°)–methanol–water (10:8:2, by vol.) solvent system for 30 hr. gave a clear separation of oestradiol-17β and oestradiol-17α.

The separation of oestradiol-17α and oestradiol-17β was achieved also by column partition chromatography. Benzene–hexane (45:55, v/v) was used for the mobile phase and methanol–water (70:30, v/v) supported on Celite for the stationary phase.

Mixtures such as oestrone, oestradiol-17β, 16-*epi*oestriol and oestriol were separated on a single paper chromatogram in a benzene–methanol–water (10:7:3, by vol.) solvent system. This method obviated the need for two separate chromatograms as described by Mitchell & Davies (1954).

Partition chromatography on a single column was used to separate oestrone, oestradiol-17α, oestradiol-17β, 16-*epi*oestriol and oestriol. The stationary phase was methanol–water (7:3, v/v) and the mobile phase was changed in discrete stages from benzene–hexane (45:55, v/v) to ethylene dichloride. The oestrogens were eluted in the sequence given above.

The application of these techniques to the study of the biosynthesis and metabolism of ^{14}C-labelled oestrogens by isotope dilution was described.

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Observations on the Formation of Bile Acids *in vitro* and *in vivo*

By Henry Danielsson (introduced by S. Bergström). (*Department of Chemistry, Karolinska Institutet, Stockholm, Sweden*)

The quantitative aspects of the conversion of cholesterol into bile acids as well as the further metabolism of bile acids in different species have been extensively studied in recent years. The sequence of reactions in the degradation of cholesterol to bile acids is, however, less well

known. From the results of studies of the metabolism of hypothetical intermediates in bile-fistula rats Bergström & Lindstedt (see Bergström, Danielsson & Samuelsson, 1960) have concluded that the transformations of the steroid nucleus precede the completion of the side-chain degradation and that an early step is the introduction of the 7α-hydroxyl group.

These investigations have recently been continued and extended by studies of the metabolism *in vitro* of cholesterol and related sterols with the aim to isolate and identify intermediary products. Labelled cholesterol was found to be oxidized by liver mitochondrial preparations to a number of different compounds, many of which must have arisen by non-enzymic oxidation (Danielsson, 1960). The main enzymically formed product was identified as 26-hydroxycholesterol (Danielsson, 1961*a*) in agreement with the work of Fredrickson & Ono (1956). This compound was converted by the bile-fistula rat into chenodeoxycholic acid and the two 6β-hydroxylated metabolites of this acid. Labelled 7α-hydroxycholesterol was converted by liver homogenates into 7α-hydroxycholest-4-en-3-one, cholest-5-ene-3β:7α:26-triol and a compound tentatively identified as 7α:26-dihydroxycholest-4-en-3-one (Danielsson, 1961*b*, *c*). These compounds were further metabolized by the bile-fistula rat into chenodeoxycholic acid and to some extent into cholic acid. Thus, the products isolated from incubations of cholesterol and 7α-hydroxycholesterol with cell-free liver preparations are precursors mainly of chenodeoxycholic acid. However, rat bile contains cholic acid and chenodeoxycholic acid in the proportion 8:2 and when labelled cholesterol is administered to a bile-fistula rat these acids become labelled in the same proportion. Assuming that the reactions observed *in vitro* correspond to those *in vivo*, the possibility emerges that the introduction of the 12α-hydroxyl group might be an early step and that the formation of cholic and chenodeoxycholic acids follow separate pathways. To test this possibility labelled 12α-hydroxycholesterol has been administered to rats and rabbits with bile-fistula and the labelled products in bile have been investigated. One of the labelled acids formed has been identified as cholic acid in both rat and rabbit.

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The Effect of 11-Deoxycorticosterone on Carbohydrate Metabolism in *Trichophyton rubrum*

By J. D. Townsley, F. W. Chattaway and A. J. E. Barlow. (*Department of Biochemistry, University of Leeds; Department of Dermatology, Royal Infirmary, Huddersfield*)

11-Deoxycorticosterone (DOC) is the most active of a group of steroids and related compounds in inhibiting the growth of four species of dermatophytes (Chattaway, Townsley & Barlow, 1959). A more detailed study of the effect of DOC upon *T. rubrum* is now reported. DOC reduces the rate of endogenous respiration and the rate of uptake of glucose by mycelium.

The effect of DOC (7·6 mM) on the incorporation pattern of ^{14}C from [^{14}C]glucose has also been studied by a chromatographic method similar to that of Moses (1958). In normal mycelium incorporation of radioactivity into 40 compounds occurs within 3 min. of addition of the [^{14}C]glucose and 8 of these are considered to be phosphorylated. After 30 min. incubation such phosphorylated compounds account for 16·3% of the ^{14}C incorporated. The remaining radioactivity was concentrated principally in fructose (67·7%), alanine (13·1%) and an unidentified compound X (1·0%). In the presence of DOC the number of phosphorylated compounds was reduced to 3 and these account for only 3·6% of the ^{14}C incorporated after 30 min. incubation. This trend was apparent from the earliest incubation period investigated (10 sec.). The major part of the ^{14}C was again incorporated into the same three compounds but the relative activities showed marked alteration. Fructose, alanine and compound X represented 51·4, 27·0 and 16·9% of the total incorporation, respectively. No evidence was found for the release by DOC of labelled compounds into the medium.

These findings bear close resemblance to those of Moses (1958) and Moses & Smith (1960) in their work on the effect of uncoupling agents on microorganisms. DOC appears to uncouple oxidative phosphorylation but these findings do not appear to be in agreement with those of Lester & Hechter (1958) who concluded that the action of DOC on *Neurospora crassa* was concerned with the binding of univalent ions rather than the energy-requiring active transport of ions across cell membranes.

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Preparation of *allo*Cholic Acid free from Cholic Acid

By I. G. Anderson and G. A. D. Haslewood. (*Guy's Hospital Medical School, London, S.E.* 1)

It is now clear that *allo* (5α) bile acids may be of importance as species characters and as clues to evolutionary history, and we have undertaken their preparation.

We previously made *allo*cholic acid by hydrogenating methyl 3α:12α-diacetoxy-7-oxocholen-5-enate. This process gives mainly cholic acid (5β) derivatives, but *allo* compounds are also formed. We now find that complete separation of these from the 5β-compounds is impracticable. Although material identical with esters of Ohta's acid is obtained, as reported (Anderson & Haslewood, 1960), it still contains cholic acid esters which cannot be removed by any method known to us.

We have therefore made *allo*cholic acid by the route: ethyl 6α-bromo-3α:12α-diacetoxy-7-oxocholanate $\xrightarrow{\text{hot OH}^-}$ 3α:7β:12α-trihydroxy-6-oxo*allo*cholanic acid [I, m.p. 219° (decomp.), $[\alpha]_D + 50°$] $\xrightarrow[\text{thioketal, Raney Ni}]{}$ 3α:7β:12α-trihydroxy*allo*cholanic acid [II, m.p. 252° (decomp.), $[\alpha]_D + 61°$] $\xrightarrow{N\text{-bromosuccinimide}}$ 3α:12α-dihydroxy-7-oxo*allo*cholanic acid (not cryst.) $\xrightarrow{NaBH_4}$ (II) + *allo*cholic acid (III). Acid (I) is entirely free from 5β-isomers, and (II) and (III) can easily be separated. A similar route, starting from the known 3α:7β-dihydroxy-6-oxo*allo*cholanic acid, is expected to give 3α:7β- and 3α:7α-dihydroxy-*allo*cholanic acids: it has already yielded, as a by-product, 3α-hydroxy*allo*cholanic (*allo*lithocholic) acid. *allo*-Cholic acid, now obtained pure, is apparently identical with the supposed 'isomer' from snakes of the genus *Bitis* (Haslewood, 1961).

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The Accumulation of α-Alanine in the Housefly, *Musca vicina*

By G. M. Price. (*Department of Biochemistry, Agricultural Research Council, Pest Infestation Laboratory, Slough, Bucks.*)

As a continuation of studies previously reported (Price, 1961) the effect of anaerobiosis on the amino acid metabolism of the housefly has been investigated.

Flies were completely immersed in boiled water at room temperature for periods up to 2 hr. (Flies recover completely even after immersion for 7 hr.) They were then killed by plunging them into liquid nitrogen and extracted either with 50% aqueous ethanol–N-formic acid or with 2% tungstic acid. Portions of the former extract were chromatographed two-dimensionally in phenol–0·1% NH_3 (Consden, Gordon & Martin, 1944) and in butanol–acetic acid–water (5:1:4, by vol.) (Phillips, 1949). The amount of α-alanine was measured after treating the chromatograms with ninhydrin. The amount of pyruvate in the latter extract was measured, as its dinitrophenylhydrazone, by the method of Tauber (1955).

There was a steady increase in the α-alanine content of the flies over the 2 hr. at a rate of 6·3 μmoles/g. of tissue/hr., this being an increase of 200%/hr. on the amount present in the 'aerobic' flies. It was also found that there was an accumulation of pyruvate in the 'anaerobic' flies.

When the two-dimensional chromatograms were compared visually there was no obvious difference in the amounts of any one of the amino acids over the 2 hr. period, apart from those of α-alanine. A similar comparison of the electrophoresis strips did not show any obvious variation in the amounts of the α-oxoglutarate and oxaloacetate hydrazones. These results suggested that transamination between aspartate and pyruvate, giving α-alanine and oxaloacetate, could account for only a very small amount of the α-alanine produced and that it must thus be formed as a result of some other reaction, such as a combination of the following two reactions:

$$\alpha\text{-oxoglutarate} + \text{DPNH} + NH_3 \rightleftharpoons \text{glutamate} + \text{DPN}$$

$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \alpha\text{-oxoglutarate} + \alpha\text{-alanine}$$

On addition,

$$\text{pyruvate} + \text{DPNH} + NH_3 \rightleftharpoons \alpha\text{-alanine} + \text{DPN}$$

It will be seen that in respect of the oxidation of DPNH to DPN the above reaction is similar to that catalysed by α-glycerophosphate dehydrogenase. In the oxidation of DPNH and in the removal of pyruvate the reaction is similar to that catalysed by lactic dehydrogenase.

Since pyruvate is the immediate precursor of α-alanine, then if this amino acid accumulates during anaerobiosis, pyruvate is of necessity removed. This is a possible explanation why the combined amounts of α-glycerophosphate, lactate and pyruvate, accumulating in cockroach muscle under anaerobic conditions, are not in stoicheiometric agreement with the amount of hexose diphosphate utilized (Chefurka, 1958).

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An Effect of Hypoxia on the Cardiac Glycogen in Rabbits

By E. C. Gordon-Smith and R. V. Coxon. (*University Laboratory of Physiology, Oxford*)

There is long-standing evidence (see Evans, 1934) that the glycogen content of the animal's heart is reduced following exposure to oxygen-lack, and it has recently been suggested that the exhaustion of such reserves may be a limiting factor in the survival of foetal and newborn animals subjected to hypoxia (Dawes, Mott & Shelley, 1959). Such depletion of the store of glycogen in the heart might be expected to lead to a subsequent increase in the rate of synthesis of this material in that organ and this might be reflected under suitable experimental conditions in an increased incorporation of ^{14}C into the cardiac glycogen. Such a sequence of events has been observed in animals surviving hypoxic episodes induced by respiratory depression under anaesthesia and also by breathing gas mixtures containing only 10% oxygen. The animals used were adult rabbits. The hypoxic periods lasted about 5 min. and were followed by an intravenous tracer dose of 20 μc of ^{14}C as uniformly labelled glucose, after which animals were sacrificed at intervals of 1½, 3 and 5 hr. The glycogen was isolated from pieces of ventricle excised while the heart was still beating and digested immediately in aqueous potassium hydroxide; after purification it was combusted and its specific radioactivity determined by the method of van Slyke, Steele & Plazin (1951). The glycogen from animals which had been rendered hypoxic was found to be 12 to 20 times more heavily labelled than that from control animals, the highest ratio being found at the longest interval after the treatment. This strongly suggested that the rate of glycogen synthesis had remained elevated for 5 hr. following an exposure to hypoxia lasting 5 min. Since this effect of hypoxia might well be, at least in part, mediated through the glycogenolytic effect of endogenous adrenaline, the influence of this hormone on incorporation of ^{14}C into cardiac glycogen was also investigated. Doses of 150 μg. subcutaneously repeated at hourly intervals produced results similar to but less intense than those of the hypoxic stimulus used in our experiments.

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Oxidative Metabolism in the Perfused Rat Heart

By J. R. Williamson (introduced by H. A. Krebs). (*Department of Biochemistry, University of Oxford*)

The saline-perfused rat-heart preparation of Bleehen & Fisher (1954) has been used to study the effects of diet and insulin on the utilization of glucose, lactate, pyruvate and acetate by the beating heart performing no external work. Changes in the concentration of metabolites in the medium and of glycogen in the heart were measured. The contribution of different metabolites to the total fuel of respiration has been calculated on the assumptions that the oxygen consumption is little affected by the nature of the metabolite principally oxidized (Fisher & Williamson, 1961) and that virtually all the metabolite removed from the medium and not converted into lactate or glycogen is completely oxidized.

Metabolizable substrates differ in their capacity to suppress the endogenous respiration of the heart, which is largely accounted for by the oxidation of non-carbohydrates. The oxidation of glucose by hearts from well-fed rats accounted for only 24% of the total respiration in the absence of insulin. This was increased to 57% by insulin (2×10^6 units/ml.). The oxidation of lactate, pyruvate and acetate by heart from well-fed rats in the absence of insulin accounted for 50%, 95% and 80% respectively of total respiration. Insulin was without effect on the oxidation of lactate and pyruvate, but it increased the contribution of acetate to 97%. Lactate was oxidized in preference to glucose in the absence, but not in the presence of insulin. Acetate, on the other hand, almost completely suppressed the oxidation of glucose, even in the presence of insulin. Hearts from rats which had been fasted for 24 hr. oxidized less glucose, lactate and pyruvate than hearts from well-fed rats. The contribution of glucose to the fuel of respiration was reduced by fasting to 16% in the absence and to 51% in the presence of insulin, and the contributions of lactate and pyruvate were reduced to 25% and 76% respectively. The oxidation of acetate was not affected by fasting.

Previous work has shown that acetoacetate contributed 75–80% to the fuel of respiration and was oxidized in preference to glucose (Williamson & Krebs, 1961). The results show that under the test conditions carbohydrate plays a minor role in

the oxidative metabolism of the beating heart. Whether this is true for the maximally working heart remains to be investigated (see Bing, 1955).

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In vivo Liberation of Inorganic Sulphate from L-Serine *O*-[^{35}S]Sulphate

By K. S. Dodgson and N. Tudball. (*Department of Biochemistry, University College, Newport Road, Cardiff*)

Dodgson, Lloyd & Tudball (1961) have demonstrated the presence, in rat liver, of an enzyme system which is capable of liberating inorganic SO_4^{2-} from potassium L-serine *O*-sulphate. The enzyme system is mainly localized in the cell sap of the liver cell and is also present in other mammalian species and in birds and fish (Dodgson & Tudball, 1961).

It has now been shown that intraperitoneally-injected L-serine *O*-[^{35}S]sulphate is extensively desulphated by the rat. Male M.R.C. hooded rats (3 months old) were each injected with 5 μmoles of L-serine *O*-[^{35}S]sulphate and the inorganic $^{35}SO_4^{2-}$ and ester [^{35}S]sulphate contents of the urines were determined by the procedures of Dodgson & Tudball (1960). Preliminary experiments were made to check the validity of the various procedures. Most of the dose (76%) appeared in the urine as inorganic $^{35}SO_4^{2-}$ together with smaller amounts (7%) of ester [^{35}S]sulphate. Some ^{35}S was present in faeces (4%) and in the carcasses of animals killed after 48 hr. (4%). Similar results were obtained with female rats.

Micro-organisms present in the intestinal tract can liberate inorganic $^{35}SO_4^{2-}$ from L-serine *O*-[^{35}S]sulphate. However, micro-organisms probably contribute little towards the extensive desulphation which occurs *in vivo* since separate experiments showed this to be a very rapid process which is virtually complete within 6 hr. Moreover, animals receiving large oral doses of sulphasuxidine and terramycin for several days before injection, continued to eliminate more than 80% of the total urinary ^{35}S as inorganic $^{35}SO_4^{2-}$.

Paper electrophoresis of the first-voided urines of mice and guinea pigs which had been injected with L-serine *O*-[^{35}S]sulphate showed that, in these cases also, rapid desulphation of the ester was occurring.

The exact mechanism of action of the liver enzyme system has not yet been established but the present results suggest that the system may be sufficiently active *in vivo* to ensure that serine residues cannot exist in an *O*-sulphated form. The possible implications of this were discussed.

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Role of the Glyoxylate Cycle in Protozoal Glyconeogenesis

By J. F. Hogg* and H. L. Kornberg.† (*Medical Research Council, Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

Since *iso*citratase and malate synthetase are present in *Tetrahymena piriformis E* which are not able to convert fats into glycogen as well as in cells which are (Hogg, 1959), the intracellular role of these enzymes has been questioned (Reeves, Papa, Seaman & Ajl, 1961). Analysis of cells grown in chemically defined media, low in ketogenic amino acids, and of cells grown in the peptone media routinely used, showed that during growth in defined media both enzymes were formed only when acetate was present and glucose absent. Both enzymes were formed during growth on peptone, the amounts being increased further by the presence of acetate. Malate-synthetase formation in the peptone media was repressed by glucose, but this repression was partially relieved by acetate. This indicates that, as in bacteria (Kornberg, 1959; Kornberg, Collins & Bigley, 1960), the formation of these enzymes by *Tetrahymena* is influenced by the composition of the nutrient media.

The specific activities (μmoles/hr./mg. protein) of *iso*citratase and malate synthetase averaged, respectively, 0·6 and 0·9 in cells incapable of performing glyconeogenesis. Cells which synthesized 0·5 μmole of glucose/hr./mg. of protein from fats contained these enzymes in higher activity: the specific activities averaged, respectively, 2·0 and 4·7. In cells capable of performing glyconeogenesis, *iso*citratase and malate synthetase were

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exclusively present in one of the two large-granule fractions obtained after disruption of the cells in 0·25M-mannitol, whereas in cells incapable of so doing, the enzymes were found in both fractions and in solution. In either case, the citrate-forming condensing enzyme was found in both large-granule fractions. This suggests that the conversion of fats into glycogen by Tetrahymena necessitates both the presence of high levels of glyoxylate cycle enzymes and their incorporation into an organized intracellular structure.

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The Effect of Thiouracil on the Metabolism of L-Thyroxine

By EUNICE V. FLOCK and JESSE L. BOLLMAN (introduced by BARBARA H. BILLING). (*Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.*)

Thiouracil, in addition to blocking the formation of thyroxine (Franklin, Lerner & Chaikoff, 1944), may alter the peripheral utilization of thyroxine (Stasilli, Kroc & Edlin, 1960). Thus, thyroxine produced only 7% as much calorigenic action in rats fed thiouracil for 6 to 8 weeks as in normal rats. Excretion of radioactive iodide from [^{131}I]-thyroxine was decreased in the urine of rats fed propylthiouracil, but excretion of thyroxine was increased in faeces (Hogness, Wong & Williams, 1954; Jones & van Middlesworth, 1960) and in bile (VanArsdel & Williams, 1956).

In a further study of the peripheral action of thiouracil on the metabolism of L-thyroxine, we have used paper chromatography and enzymic hydrolysis for examining the metabolites of thyroxine in bile, urine, and plasma of rats which had received a diet, 0·1% of which was thiouracil, for 2–10 weeks prior to the intravenous injection of thyroxine labelled with ^{131}I in the 3′- or 5′-position. The relative amounts of ^{131}I excreted in bile and urine by rats which had received thiouracil in the diet for 2 weeks did not differ greatly from those excreted by rats not receiving any thiouracil. Rats fed thiouracil for 4 weeks or longer excreted definitely more ^{131}I in bile and less in urine.

Approximately two-thirds of the ^{131}I from thyroxine which was excreted in the bile by the normal rat was found in the glucuronide region on paper chromatograms. When this was incubated with β-glucuronidase, the major hydrolytic product was thyroxine with minor amounts of 3,3′,5′-tri-iodothyronine, 3,5,3′-tri-iodothyronine, 3,3′-di-iodothyronine, and tetraiodothyroacetic acid. The feeding of thiouracil for 2 weeks did not alter this. When rats were fed thiouracil for 4 weeks or longer, however, approximately one-fourth of the ^{131}I excreted in bile was in thyroxine glucuronide and one-fourth was in 3,3′,5′-tri-iodothyronine glucuronide, in contrast to 40% as thyroxine glucuronide and 6% as 3,3′,5′-tri-iodothyronine glucuronide in the normal rat. The formation of large amounts of 3,3′,5′-tri-iodothyronine, which is calorigenically inactive (Stasilli, Kroc & Meltzer, 1959), in rats fed thiouracil for 4 weeks or more may account for the decreased calorigenic action of thyroxine in these animals. 3,3′,5′-Tri-iodothyronine glucuronide has previously been found with thyroxine glucuronide in bile of dogs with livers and also in large amounts in plasma and urine of dehepatized dogs following administration of [^{131}I]thyroxine (Flock, Bollman, Grindlay & Stobie, 1961).

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Some Aspects of Intermediary Metabolism in Tissue Culture Cells

By C. BRYANT, M. J. H. SMITH, K. REYNOLDS and V. MOSES. (*Department of Chemical Pathology, and Surgical Unit, King's College Hospital Medical School, London, S.E. 5*)

The incorporation of radioactivity from [^{14}C]-glucose, [2-^{14}C]acetate and [1:4-^{14}C]succinate into the soluble metabolic intermediates of human foetal liver cells and HeLa cells in culture has been investigated. Both types of cells incorporated ^{14}C from each of the labelled substrates into hexose phosphates, lactic acid and amino acids, particularly alanine, aspartic acid and glutamic acid. With [2-^{14}C]acetate, radiocarbon was also present

in tricarboxylic acids and the amounts of isotope appearing in these acids were substantially increased when labelled succinate was used as the substrate.

The foetal liver cells oxidized more of the labelled glucose and succinate than the HeLa cells. A further difference between the two types of cells concerned the distribution of the radioactivity from the labelled succinate between the organic acids. The relative proportions of ^{14}C present in malic, citric and lactic acids were found to be 1:5:8 for the foetal liver cells and 5:1:1 for the HeLa cells. These results were discussed.

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Intermediary Metabolism in the Isolated Rat Diaphragm

By A. K. Huggins and M. J. H. Smith. (*Department of Chemical Pathology, King's College Hospital, Medical School, London, S.E.* 5)

The incorporation of ^{14}C from [^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate into the soluble intermediates of isolated rat diaphragm has been studied using Krebs–Ringer phosphate or Krebs–Ringer bicarbonate (Umbreit, Burris & Stauffer, 1957) as incubation media. The radioactive substances in the tissue extract and in the incubation medium were separately analysed by paper chromatography and radioautography (Smith & Moses, 1960).

The relative proportions of radioactivity in the soluble intermediates of the separated medium and of the tissue extract were found to vary both with the labelled substrate and with the incubation medium. Using Krebs–Ringer phosphate the ratios of ^{14}C between medium and tissue were as follows: [^{14}C]glucose, 10; [3-^{14}C]pyruvate, 4 and [2-^{14}C]acetate, 1; whereas, with Krebs–Ringer bicarbonate the corresponding values were 3, 3 and 0·5 respectively.

The qualitative pattern of incorporation of radioactivity from the three labelled substrates was very similar in both incubation media. The ^{14}C occurred in substances known to be involved in glycolysis, the tricarboxylic acid cycle, transamination and synthetic reactions. However, when labelled glucose was used as the substrate a greater proportion of radioactivity was found in the amino acid fraction when Krebs–Ringer phosphate was the incubation medium. The effects of 2:4-dinitrophenol and salicylate on the incorporation and subsequent distribution of ^{14}C from the labelled substrates into the soluble intermediates of isolated rat diaphragm were discussed.

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An *in vitro* Effect of Vitamin B_{12} on the Oxidation of Glutathione in Erythrocytes

By P. C. Jocelyn. (*Department of Biochemistry, University of Edinburgh*)

When washed erythrocytes are oxygenated, the rate of oxidation of glutathione in the cells depends on the constitution of the suspending medium. Using saline or human dialysed serum or plasma (HDP) as the medium, the rate of oxidation of glutathione is increased by Cu^{2+} (50–200 μg. %), SeO_2 (100–400 μg. %) and ascorbic acid (100 mg. %).

When human dialysed serum or plasma is obtained by a short dialysis (e.g. 24 hr.) against copper-free saline, the rate of oxidation of glutathione in the cells is often inhibited by vitamin B_{12} added at concentrations of 100–10 000 $\mu\mu$g./ml. of medium. If this inhibition is considerable, Cu^{2+} (25–100 μg. %) subsequently added, neutralizes the effect of the vitamin. On the other hand, when the inhibition is small, the increase in rate of oxidation of glutathione induced by Cu^{2+} is neutralized by the vitamin. Addition of vitamin B_{12} to haemolysates does not produce any inhibition.

The results suggest that the vitamin may inhibit the entry of Cu^{2+} into erythrocytes.

Spectrophotometric Methods for the Determination of Succinic-Dehydrogenase and Succinic-Oxidase Activities in Mitochondrial Preparations

By E. R. Redfearn and Jean M. Dixon. (*Department of Biochemistry, University of Liverpool*)

The reduction of phenazine methosulphate by succinate in mitochondrial preparations can be coupled to 2:6-dichlorophenolindophenol and the rate of reduction of the latter measured spectrophotometrically (Ells, 1959). This reaction has been investigated in further detail.

Pig-heart-muscle preparations (Pumphrey & Redfearn, 1960) were used. Succinic dehydrogenase activity was measured by the rate of reduction of 2:6-dichlorophenolindophenol at 600 mμ using

a series of concentrations of phenazine methosulphate. The double-reciprocal plot of the activity concentration curve gave a straight line for concentrations less than a certain value (66–136 μmoles of phenazine methosulphate/mg. of enzyme protein depending on the age of the preparation) above which phenazine monosulphate was inhibitory. Extrapolation of the straight portion of the curve gave the maximum activity for infinite concentration of phenazine methosulphate.

In order that the succinic oxidase activity could be measured under comparable conditions, the following method was devised. The reaction mixture containing cytochrome *c* was placed in a spectrophotometer cell. The cytochrome *c* remains in the oxidized form until all the oxygen in the mixture has been utilized. The cytochrome *c* then immediately begins to be reduced and this can be detected by the sudden rise in extinction at 550 mμ. Thus, by observing the time between the start of the reaction and the onset of cytochrome *c* reduction and calculating the concentration of oxygen in the mixture at the temperature of the assay, the succinic oxidase activity could be determined. Parallel determinations by polarographic and manometric methods gave very close agreement.

The succinic dehydrogenase and succinic oxidase activities were determined in a heart-muscle preparation (stored at $-20°$) over a period of 23 days. It was found that the succinic dehydrogenase activity declined steadily as the preparation aged whereas succinic oxidase remained at a fairly constant level of activity. The ratio succinic dehydrogenase/succinic oxidase was as high as 4·9 in the fresh preparation but declined to 2·0 at the end of 23 days. These ratios are much higher than those obtained by Giuditta & Singer (1959) using manometric methods. The explanation for the difference may be that reduced phenazine methosulphate reacts much faster with 2:6-dichlorophenolindophenol than it does with oxygen.

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The Fractionation of Russell's Viper Venom with Special Reference to the Coagulant Protein

By W. J. Williams and M. P. Esnouf. [*Nuffield Department of Clinical Biochemistry* (*University of Oxford*), *Radcliffe Infirmary, Oxford*]

It has been known for many years that Russell's viper venom has coagulant activity (Macfarlane & Barnett, 1934). Recently Grasset & Schwartz (1955) fractionated the viper venom by electrophoresis on paper strips. They recovered a fraction which contained 80% of the coagulant activity of the crude venom. This fraction also possessed gelatinase activity.

We have fractionated the venom by chromatography on columns of diethylaminoethylcellulose. The chromatograms were developed at room temperature by stepwise elution with 0·01M-2-amino-2-hydroxymethylpropane-1:3-diol–phosphate buffers at pH 8·5, 7·0, and 6·0, and then at pH 6·0 with 0·15M-, 0·5M- and M-NaCl. The main bulk of the coagulant activity was recovered in the fraction eluted with 0·15M-NaCl. This fraction was electrophoretically homogeneous, but contained two components with different sedimentation constants. These components were separated by centrifugation at 100 000*g* for 40 hr. into a sucrose gradient (7–20% sucrose) at 3°. The coagulant protein obtained in this way was homogeneous in the ultracentrifuge and by electrophoresis.

The specific activity of the coagulant protein obtained from the columns was increased about 12-fold as compared with the crude venom. There was some loss of activity during the subsequent steps which resulted in a final purification of about eightfold.

The purified protein reacts with a bovine-serum fraction in the presence of ionized calcium to produce a powerful coagulant activity (Macfarlane, 1961). The coagulant activity generated in this way is independent of the amount of the venom protein added over a tenfold range, but is directly related to the amount of serum fraction added, suggesting that the venom protein is acting as an enzyme and the serum fraction as the substrate. Maximum activity could be obtained over a pH range from 7·0 to 9·0.

The purified coagulant protein was tested for the various enzymic activities which are known to be present in the crude venom. The only enzymic activities which have been detected were an esterase activity against toluene-*p*-sulphonylarginine-methyl ester, and a slight gelatinase activity.

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Thymidine Kinases in Normal and Malignant Tissues

By P. A. Bianchi, A. R. Crathorn and K. V. Shooter. (*Chester Beatty Research Institute, Fulham Road, London, S.W.* 3)

It has been demonstrated that the enzymes which convert thymidine into thymidine triphosphate

(TTP), a necessary step in the incorporation of thymidine into DNA, occur in the cytoplasm of many systems of rapidly dividing mammalian cells (Bollum & Potter, 1959; Mantsavinos & Canellakis, 1959; Smellie, Keir & Davidson, 1959; Weissman, Smellie & Paul, 1960; Bianchi, Butler, Crathorn & Shooter, 1961). Bollum & Potter (1959) suggested that the phosphorylation reactions occurred in the sequence T → TMP → TTP but Weissman *et al.* (1960) have maintained that thymidine diphosphate (TDP) is an intermediate in the formation of TTP. We have studied the time course of the phosphorylation with enzyme systems from several tissues and cells and in no case has TDP been detected until considerable amounts of TTP have been formed. At high dilutions of the enzyme system when the rates of the successive reactions controlled by diffusion processes are slowed down, TDP does not appear at all. The sequence of reactions proposed by Bollum & Potter (1959) would therefore appear to be correct. Further experiments indicate that TDP is formed by dephosphorylation of TTP.

Further studies of various tissues have shown that there is some correlation between mitotic activity and the content of thymidine kinases (Bollum & Potter, 1958, 1959). We have studied the kinase activity during the growth of two mouse tumours to see if any variations in activity with time can be detected. In the first experiments Swiss S mice were injected intraperitoneally with Ehrlich ascites cells, and groups were killed after 2, 4, 6, 8 and 10 days. The kinase activity per cell after two days was lower than that of the cells used for injection, the activity then rose to a maximum at 4 days and decreased rapidly during the next 6 days. In the second experiment Akl mice were injected intraperitoneally with a spleen homogenate from similar mice with a virus-induced leukaemia (Miller, 1960). Groups of the mice were killed after 2, 4, 6, 8 and 10 days and the kinase activity of the spleen cells was determined. After 2 days the ability to convert thymidine into TMP was reduced and only a very small part of the TMP formed was further phosphorylated to TTP. After 4 days the kinase activities were found to be higher than those of spleens from uninjected mice; a further increase in activity had occurred by 6 days and this was little changed after 8 and 10 days.

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Thymidine Kinases in Human Tumours

By P. A. Bianchi. (*Chester Beatty Research Institute, Fulham Road, London, S.W.* 3)

It has been shown that particle-free fractions prepared from many rapidly dividing mammalian tissues by homogenization and high-speed centrifuging contain enzymes which are capable of phosphorylating thymidine to the triphosphate (TTP) and also the enzyme which polymerizes the triphosphates of the four nucleosides into DNA (Bollum & Potter, 1958). In the animal tissues studied the sequence of reactions appears to be T → TMP → TTP → DNA. The enzyme activities of particle-free fractions from various human tissues have now been studied (normal lymph nodes, lymph nodes from a patient with lymphosarcoma, three samples of leucocytes from patients with myeloid leukaemia, three samples of leucocytes from a patient with acute leukaemia and a sample of a spleen from a patient with hereditary spherocytosis). When fractions from these tissues were incubated with 4 μm-moles of [^{14}C]thymidine and an ATP-regenerating system (Bollum & Potter, 1958) the rate of production of TTP was found to be much slower than had been observed with fractions from normal and malignant mouse tissues. After incubation for 30 min. the amount of thymidine converted into TTP ranged from 4% with the proprotein from normal human lymph nodes to 30% for one fraction from the leucocytes of a myeloid leukaemia. In these experiments no TMP could be detected on the chromatograms. Since this suggested that any TMP formed was converted into TTP very quickly the fractions were incubated with 4 μm-moles of [^{14}C]TMP. It was found that the fractions from all the human tissues could convert the added TMP completely into TTP in less than 30 min. It is clear therefore that the human tissues studied must contain only very small amounts of the enzyme which phosphorylates thymidine to TMP. These observations are in marked contrast to those obtained from studies on tissues from rats and mice, which in general possess a high content of the enzyme producing TMP. The physiological significance of this difference in the enzyme content of human tissues and those from rats and mice is not clear, particularly since thymidine is not a normal metabolite. The absence of the enzyme producing TMP may perhaps explain the apparently anomalous

results recently reported by Gavosto, Maraini & Pileri (1960) who used thymidine incorporation to assess the proliferative capacity of human leukaemic cells.

Assays of the polymerase of the human tissues have shown that the enzyme content roughly parallels the mitotic activity. Both the polymerase and the thymidylate activity of leucocytes of myeloid and of acute leukaemia are reduced after prednisone treatment.

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Charge-Transfer Complexes of Lipoyl Dehydrogenase

By V. Massey and G. Palmer. (*Department of Biochemistry, University of Sheffield*)

Oxidized lipoyl dehydrogenase is yellow because of its flavin prosthetic group. Anaerobic reaction of the pig-heart enzyme with DPNH or dihydrolipoyl derivatives produces the red half-reduced form which is an important intermediate in catalysis (Massey, Gibson & Veeger, 1960). When the reduction with DPNH is carried out in the presence of arsenite, the red form is converted into a blue-green species, characterized by a broad absorption band (500–1000 mμ). This same band is also produced in the absence of arsenite when the reaction is carried out in ice and with copper-inactivated enzyme (Massey & Veeger, 1961). These spectral changes are produced only in the presence of pyridine nucleotides; never by dihydrolipoyl compounds.

Neurospora diphosphopyridine nucleotidease (DPNase) hydrolyses DPN but does not attack DPNH. When added to the green form of lipoyl dehydrogenase it causes disappearance of the green band; the resulting spectrum is that of fully reduced enzyme. The addition of DPN results in the immediate reappearance of the green band, which gradually disappears as the added DPN is destroyed by the DPNase. Entirely similar results are obtained when deamino-DPNH is employed instead of DPNH; however the rate of disappearance of this band on the addition of DPNase is much slower than when DPNH is used. This is in keeping with its much lower rate of hydrolysis of deamino-DPN. Once full reduction of lipoyl dehydrogenase is obtained, the addition of deamino-DPN again produces the green band. The absorption maximum with both the DPN and the deamino-DPN systems is at 700 mμ.

When the experiments were repeated with thionicotinamide DPNH (TNDPNH) the formation of the green band was much slower and the maximum was at 740 mμ. An intermediate with an absorption maximum at about 600 mμ was formed in this reaction, disappearing as the final green form was produced. The destruction of the green form by DPNase was very slow, in agreement with its specificity, and addition of TNDPN to the fully reduced enzyme immediately regenerated the long wavelength band, without any detectable formation of the 600 mμ intermediate.

In view of the broad structureless nature of the absorption spectra, temperature dependence and the variation of the wavelength maximum in the expected way with variation in redox potential of the pyridine nucleotide, it is proposed that the green form is due to a charge-transfer complex between $FADH_2$ and oxidized pyridine nucleotide (see Kosower, 1960). Similar complexes have also been studied in model systems employing $FMNH_2$ and *N*-methylnicotinamide.

Contrary to the currently popular view of the biological importance of charge-transfer complexes, the slow rate of formation and production only under conditions detrimental to physiological activity indicate that they do not play any important role in the catalysis of this enzyme.

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Interactions between Serum Proteins and Detergents in Agar Gels

By M. P. Tombs and R. D. Weston. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

In immuno-electrophoresis, one of the most powerful methods of qualitative protein analysis at present available, proteins are differentiated on the basis of charge and rate of diffusion through agar. Immunological specificity is also a factor, although not always a decisive one, the antisera being employed more as general precipitating reagents; for some materials antisera are difficult to obtain. The precipitating characteristics of antibodies are similar to those of detergents, which are powerful protein precipitants, in many cases redissolving the precipitate in excess. Detergents would be expected therefore, in appropriate conditions, to form sharp

lines of precipitate in diffusion plates. We have been able to demonstrate that they do behave similarly to antisera in this respect.

The position of the lines between sample and detergent wells varied with concentration of sample and detergent in a way similar to that seen with antisera. Hydrogen-ion concentration proved to be critical and had to be carefully controlled. By varying the pH and the detergent some specificity of precipitation was obtained.

In experiments with serum proteins, anionic detergents were found to be most effective at pH 4–5 (Teepol, and sodium dodecylbenzenesulphonate (SDBS)) and cationic at pH 8 (cetylpyridinium chloride and cetyltrimethylammonium chloride). In gels where electrophoresis was first carried out at pH 8·6 the pH of the gel was subsequently adjusted by immersion in a bath of a suitable buffer for 20 min. In this time the diffusion of the proteins was negligible. By using a triangular arrangement of three wells it was possible to investigate the effect of one protein on the diffusion and precipitation of another by detergents. It was shown that albumin inhibits the precipitation of γ-globulin by sodium dodecylbenzenesulphonate. It appeared to do this by competing with γ-globulin for the available detergent, although it was not itself precipitated. Non-ionic detergents (Tween 80 and 20) inhibited precipitation but polyethylene glycol (Carbowax 20M) had comparatively little effect. Results similar to proteins were obtained with chondroitin sulphate, and with nucleic acids diffused against histones.

It was possible to determine diffusion constants by a slight modification of the angle-plate technique (Allison & Humphries, 1960). The apparent diffusion constant of sodium dodecylbenzenesulphonate varied from about 40×10^{-7} at pH 4 to 7×10^{-7} at pH 6, suggesting that detergent micelles were involved in the precipitation reactions, and that their size depended on pH.

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Tyrosine Phosphorescence of Proteins

By J. W. Longworth (introduced by G. Weber). (*Department of Biochemistry, University of Sheffield*)

Phenylalanine, tyrosine and tryptophan are the only amino acids that give a phosphorescence (Debye & Edwards, 1952; Steele & Szent-Györgyi, 1958). These studies, and the high resolution spectra of Vladimirov & Litvin (1960), suggested that the phosphorescence of proteins was due only to the tryptophan residues. A similar conclusion has previously been reached as regards protein fluorescence (Teale, 1960), but more recently a small tyrosine component has been detected (Teale, 1961; Vladimirov, 1961; Weber, 1961).

The experiments presented show that protein phosphorescence consists of two bands: a low-intensity, structureless band, maximum 388 mμ, similar to that of tyrosine, and a more intense structured band with maxima at 410 mμ, 436 mμ, 460 mμ, which is similar to that of tryptophan. The tyrosine component is increased relatively to the tryptophan by urea, acid or propylene glycol.

By means of a rotating specimen phosphoroscope, cooled to 77° K, the lifetimes and excitation spectra of both phosphorescence bands of a protein have been studied. A complete separation of the two emissions was possible by the use of broad-band light filters; a Chance OX 1 transmits only the tyrosine phosphorescence, while a Chance OY 3 passes only the tryptophan band.

The lifetimes of phosphorescence of phenylalanine, tyrosine and tryptophan are 7·3, 2·1 and 5·3 sec. respectively, in agreement with the values of Steele & Szent-Györgyi (1957). The lifetimes for OX 1 phosphorescence was $2{\cdot}1 \pm 0{\cdot}1$ sec. for bovine-serum albumin, chymotrypsinogen, lysozyme and insulin. The OY 3 phosphorescence had a lifetime of $5{\cdot}3 \pm 0{\cdot}1$ sec. for bovine-serum albumin and chymotrypsinogen, but only $1{\cdot}2 \pm 0{\cdot}05$ sec. for lysozyme. No evidence for any phenylalanine phosphorescence could be detected.

The excitation spectra of the bovine-serum albumin phosphorescence bands have been determined. The OY 3 band has the excitation spectrum of tryptophan, and the OX 1 band a spectrum similar to that of tyrosine. In acid or propylene glycol, the excitation spectrum of the OX 1 band is essentially identical with that of tyrosine, while at pH 7·0 the band width is greater, the increase being due to a shift of the long wavelength of the band. Addition of sucrose to neutral solutions was investigated, since sucrose causes shifts to longer wavelengths of the absorbancy of 'exposed' tyrosine residues (Herskovits & Laskowski, 1960). The excitation spectrum has a decreased band width from the neutral solution, the short-wavelength edge and the peak shift to longer wavelengths, though the long-wavelength edge remains unchanged. These results can be interpreted by the occurrence of two types of tyrosine environments in the native protein and only one in the acid- or solvent-treated protein. The shorter-wavelength-absorbing tyrosine residues of the native protein would appear to be in 'exposed' environments.

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Conduction of Protons through the Membranes of Mitochondria and Bacteria by Uncouplers of Oxidative Phosphorylation

By P. MITCHELL. (*Department of Zoology, University of Edinburgh*)

According to the chemiosmotic hypothesis (Mitchell, 1960, 1961*a*, *b*), there are three main components of the organelles that catalyse oxidative phosphorylation: an oxido-reduction system catalysing electron translocation; an adenosine triphosphatase system catalysing the reversible translocation of OH′ ions from inorganic phosphate; and a charge-impermeable membrane in which the oxido-reduction and adenosine triphosphatase systems are orientated in opposition, so that the movement of electrons across the membrane during oxido-reduction must push a corresponding number of OH′ ions from inorganic phosphate, thus generating phosphorylium for donation to adenosine diphosphate. If this hypothesis were correct, one might reasonably suppose that uncouplers of oxidative phosphorylation could act by making the membrane permeable to charge.

It has been discovered, by means of a simple titration technique, that the normally very low rate at which protons diffuse through the membranes of washed suspensions of *Micrococcus lysodeikticus* (Gilby & Few, 1958) and of rat-liver mitochondria isolated as described by Myers & Slater (1957) is increased manyfold by the addition of dicoumarol, 2:4:dinitrophenoxide, azide, and other uncouplers of oxidative phosphorylation. At low concentrations of uncouplers of the dinitrophenol type, the rate of conduction of protons across the membrane is proportional to the amount of uncoupler added. The concentrations of the agents which give nearly complete uncoupling (e.g. for the mitochondria: 20 μM-dicoumarol; 100 μM-dinitrophenoxide; 2mM-azide; for the bacteria: 10 times the concentrations of the same agents) have been found to catalyse the passage of protons through the membranes of resting mitochondria or bacteria at rates equivalent to the rates of passage of the electrons through the respective oxido-reduction systems during uncoupled glutamate oxidation.

These observations are entirely consistent with the chemiosmotic coupling hypothesis which they were designed to test. They also imply that the dinitrophenol type of uncoupling agent owes its activity to two factors, (*a*) solubility in a non-polar region in the membrane of the phosphorylation system (Shaw, Lannon & Tapley, 1959), and (*b*) the capacity for conducting protons across this region. The latter capacity can possibly be ascribed most simply to the presence of two or more alternative weakly acidic groups between which the proton-bonding electron can pass by way of the π-orbitals present in all uncouplers of this type.

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The Action of Ribonuclease on the Microsomal Particles of Reticulocytes

By H. R. V. ARNSTEIN. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Several reports indicate that the microsomal ribonucleoprotein particles of various cells are relatively resistant to ribonuclease, especially at low temperatures (Tissières & Watson, 1958; Ts'o, Bonner & Vinograd, 1958; Shigeura & Chargaff, 1960), and it has been postulated that dissociation of the ribonucleoprotein is a prerequisite for attack by the enzyme (Shigeura & Chargaff, 1960).

In the present work, microsomal particles obtained from rabbit reticulocytes (Schweet, Lamfrom & Allen, 1958) were suspended in medium *A* (0·35M-sucrose, 0·035M-$KHCO_3$, 0·004M-$MgCl_2$, 0·025M-KCl) (approx. 10 mg./ml.) and incubated at 13–15° for 30 min. with pancreatic ribonuclease (0·3 μg./ml.). After cooling to 4°, the incubation mixture was diluted with medium *A* (4–12 vol.) and centrifuged at 100 000*g* for 60 min. The pellets were washed by resuspending in medium *A* (13 ml.), and the suspension was centrifuged as before. Treatment with ribonuclease resulted in some degradation of RNA as shown by release of u.v.-absorbing material which did not sediment with the microsome pellets, but this was relatively small (approx. 20% of the total RNA compared with 8% in the controls). Moreover, the RNA/protein ratio of the

washed, ribonuclease-treated particles was only slightly lower than that of untreated particles. The incorporation of radioactive valine into protein by the ribonuclease-treated microsomes under the conditions described by Schweet *et al.* (1958) was, however, only 10–20% of that obtained with control microsomes. This inhibition was not due to adsorbed ribonuclease, since addition of inactivated microsomes did not reduce the incorporation of valine by control microsomes. Similar pretreatment of the microsomes with deoxyribonuclease (10–100 μg.) had little or no effect. When suspensions of the particles in 0·1M-$NaHCO_3$–0·005M-$MgCl_2$ were sedimented in the analytical ultracentrifuge, a major peak (71–74S) and a second peak (107S) were found in both normal and ribonuclease-treated preparations, but two further minor peaks (133S and 155S) were absent after ribonuclease treatment.

These results suggest that the part of the microsomal RNA which is important for protein synthesis is particularly sensitive to ribonuclease.

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Observations on the Influence of Rat-liver Preparations on Collagen

By D. Mary Frankland and C. H. Wynn. (*Rheumatism Research Centre, University of Manchester, York Place, Manchester* 13)

The catabolism of collagen can in certain conditions (e.g. post-partum uterus) become rapid. However, the only collagenase characterized is of bacterial origin (Seifter, Gallop, Klein & Meilman, 1959) and no known tissue enzyme is capable of attacking collagen, except to a limited extent or at conditions of ionic strength and temperature differing widely from those normally expected in mammals.

Woessner & Boucek (1961) have shown that, during the development and resorption of connective tissue in the polyvinyl sponge, the activities of all enzymes studied increased during the development stage. Whereas the activities of enzymes, such as acid phosphatase, which are thought to be associated with lysosomal particles, continue to increase during the resorption phase, the activities of enzymes known to be mitochondrial in origin decrease during this phase. However, it has been reported that native collagen is not a substrate for acid cathepsin, a lysosomal enzyme (Orekhovitch, Shpikiter, Mazurov & Kounina, 1960).

A homogenate of rat liver in 0·25M-sucrose was separated by differential centrifugation into nuclear, heavy mitochondrial, light mitochondrial and microsomal fractions (De Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). After freezing and thawing ten times, the activity of each fraction towards Azocoll was determined by the release of dye, measured at 525 mμ, after incubation with Azocoll in 0·05M-acetate buffer, pH 5·0, for 1 hr. at 37°. The heavy and light mitochondrial fractions show the greatest activity when expressed as relative specific activity (i.e. % of total activity/% of total N), the distribution of activity closely resembling that of the acid phosphatase activity of the fractions.

An homogenate of rat liver, after freezing and thawing 10 times and centrifugation at 80 000*g* for 1 hr. at 1°, reduced the viscosity of a 0·1% solution of acid-soluble collagen in 0·1M-acetate buffer, pH 5·0, by 20% in 2 hr. at 35°. Suitable control experiments showed little decrease in viscosity.

Purification of the liver extract by acetone and ammonium sulphate fractionation yields a preparation which reduces the viscosity of collagen by 50% at 35° in 1 hr.

In contrast to bacterial collagenase, the activity is not inhibited by 0·01M-cysteine.

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Incorporation of [^{14}C]Leucine into Serum Albumin by the Isolated Microsome Fraction of Rat Liver

By P. N. Campbell, Barbara A. Kernot and L. F. Smith.* (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W.* 1, *and Department of Biochemistry, University of Cambridge*)

It has previously been reported that when the microsome fraction from rat liver is incubated under

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suitable conditions there is an energy-dependent incorporation of [^{14}C]amino acid into serum albumin. The labelled albumin may be released from the particles by ultrasonic vibrations (Campbell, Greengard & Kernot, 1960). Before this finding can be taken as evidence of albumin synthesis *de novo* more information is required concerning the way in which the [^{14}C]amino acid is incorporated into the polypeptide chain of serum albumin. In an attempt to resolve this problem the distribution of radioactivity among the products of the trypsin digestion of radioactive albumin has been studied.

The microsome fraction from 40 g. of regenerating rat liver was incubated for 50 min. at 37° with cell sap, phosphoenolpyruvate, pyruvate kinase and ATP in a tris-buffered medium (Rendi & Hultin, 1960) together with 120 μc of [^{14}C]leucine. The [^{14}C]leucine had a specific radioactivity of 90 mc/m-mole, and was purified by paper chromatography shortly before use. After incubation the particles were isolated by centrifuging and subjected to ultrasonics. The soluble extract obtained was freeze-dried and the serum albumin recovered by addition of phosphate buffer pH 2·4 and ethanol (Peters, 1958). The protein recovered by freeze-drying was further purified by electrophoresis on cellulose acetate, heat-denatured, oxidized with performic acid, and hydrolysed with trypsin (Hirs, 1956). The hydrolysate was then subjected to electrophoresis on paper in pyridine acetate buffer pH 3·5 at 2000 v for 2 hr. (Ryle, Sanger, Smith & Kitai, 1955). A radioautograph of the paper was then obtained.

Another sample of [^{14}C]leucine (50 μc) was injected into a rat (200 g. body wt.) and the animal was killed after 30 min. Serum albumin was isolated from the microsome fraction of the liver as described above, oxidized and the trypsin hydrolysate subjected to electrophoresis simultaneously with the albumin labelled *in vitro*.

The radioautographs from both the *in vitro* and *in vivo* labelled albumins showed approximately 20 different bands of radioactivity. Nearly all the bands from the two samples coincided but there were some differences in the relative densities of the bands from the two samples.

The results indicate that under the *in vitro* conditions used there is extensive incorporation of [^{14}C]leucine into serum albumin. This would not be so if the incorporation were limited to the exchange of a small number of amino acid residues with free [^{14}C]leucine. However, it is not clear to what extent completely new polypeptide chains are synthesized *in vitro*.

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Factors Influencing Rates of Amino Acid-Dependent Pyrophosphate Exchange

By Priscilla Hele. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London, W.* 12)

The pH 5·0 fractions (Keller & Zamecnik, 1956) obtained from the livers of 6-week-old rats catalyse a maximum rate of leucine-dependent pyrophosphate exchange at 5 mM-$MgCl_2$. This rate decreases with increasing $MgCl_2$ concentration. With isoleucine the rate increases with $MgCl_2$ concentration to a maximum at 20–30 mM-$MgCl_2$. The stimulating effect of high $MgCl_2$ concentrations on isoleucine-dependent pyrophosphate exchange is greatly reduced when fresh pH 5·0 fractions obtained from older animals (12-weeks and 8 months old) are employed, whilst leucine-dependent pyrophosphate exchange is slightly stimulated and not depressed. Transfer of [^{14}C]isoleucine and [^{14}C]-leucine to 'soluble' ribonucleic acid catalysed by these fractions is about one-third that obtained with fractions obtained from animals 6 weeks old. Addition of phenol-extracted 'soluble' ribonucleic acid (Bosch, Bloemendal & Sluyser, 1960) restores the capacity of these pH 5·0 fractions from older animals to catalyse increased rates of pyrophosphate exchange with isoleucine at high $MgCl_2$ concentrations, but has little effect on the rate measured at 5 mM-$MgCl_2$. Adding 'soluble' ribonucleic acid to pH 5·0 fractions from 6-week-old rats depresses the rates with isoleucine measured at high $MgCl_2$ concentrations. Recently, Ogata, Nohara, Ishikawa, Morita & Asaoka (1961) reported a stimulating effect of 'soluble' ribonucleic acid on pyrophosphate exchange with isoleucine as substrate in guinea-pig-liver preparations.

With fractions low in ribonucleic acid (Hele & Finch, 1960; Hele, 1960*a*, *b*) or pH 5·0 fractions incubated at 37° to remove the terminal nucleotide sequence of 'soluble' ribonucleic acid (Hecht, Stephenson & Zamecnik, 1959) these effects of increasing $MgCl_2$ concentration are reversed, for the rate with leucine as substrate is stimulated, whilst that with isoleucine is depressed. Such incubation also abolishes the stimulating or depressing effect of added 'soluble' ribonucleic acid at high $MgCl_2$ concentrations. This suggests the existence of two isoleucine-dependent pyrophosphate exchange reactions. One is catalysed by a stable system at lower

$MgCl_2$ concentrations, and is not very sensitive to 'soluble' ribonucleic acid. The other is catalysed by a labile system at high $MgCl_2$ concentrations, and is sensitive to 'soluble' ribonucleic acid. The terminal nucleotide sequence of 'soluble' ribonucleic acid may stabilize the labile component (Hele, 1960*b*).

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **409th Meeting** *of the Society was held in the School of Pharmacy, University of London, on Friday, 6 October* 1961, *and in the Westminster Medical School on Saturday, 7 October* 1961, *when the following papers were presented:*

COLLOQUIUM ON 'PHOSPHOLIPIDS AND SULPHOLIPIDS'

The Importance of Electrokinetic Potentials in Some Phospholipase–Substrate Interactions

By R. M. C. Dawson and A. D. Bangham. (*Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge*)

Two water-soluble phospholipases have been investigated: (1) the lecithin-splitting activity of *Penicillium notatum* phospholipase B, and (2) the phospholipase C (α-toxin) of *Clostridium perfringens*. The rate of enzymic hydrolysis has been related to the electrokinetic properties of the phospholipid substrate particles. The ζ-potential of such particles (e.g. lecithin micelles) has been varied by adding amphipathic molecules to the micelles (i.e. long-chain bases or acids) while the ζ-potential of both substrate and enzyme has been changed by introducing polyvalent ions, e.g. Ca^{2+} or $Fe(CN)_6^{3-}$, into the bulk buffer phase. This produced activation or inhibition of the enzymes.

Such experiments showed that *P. notatum* phospholipase required lecithin micelles to possess a minimum net negative ζ-potential before activity commenced. This could be provided by introducing long-chain acids, e.g. phosphatidylinositol, cardiolipin, into the micelle or by adding $Fe(CN)_6^{3-}$ to the bulk buffer; long-chain bases and polyvalent cations, e.g. Ca^{2+}, Th^{4+}, were inhibitory.

α-Toxin only hydrolysed lecithin when this possessed a positive ζ-potential, achieved either by adding long-chain bases, e.g. stearylamine, to the micelles or certain metals to the bulk, e.g. Ca^{2+}, UO_2^{2+}; conversely, long-chain acids and $Fe(CN)_6^{3-}$ were inhibitory. Pure α-toxin is isoelectric at pH 5·5 so it was negatively charged at the pH at which activity was normally determined. However, on using lecithin, which was negatively charged, no activity was observed below pH 5·5, i.e. with the enzyme positively charged. Phosphatidylethanolamine is negatively charged above pH 4, nevertheless, with Ca^{2+} present limited hydrolysis was detected and this was increased when the surface of the substrate was diluted with lecithin or when it was combined in a lipoprotein.

That such effects are confined to the lipid–water interface was demonstrated by following the enzymic hydrolysis of unimolecular phospholipid films near to the collapse pressure. With low-pressure films hydrolysis occurred in the absence of an activator and electrostatic requirements were not in evidence.

It is concluded that before the enzymes can hydrolyse micelles or high-pressure films of phospholipids the ζ-potential at certain points on the interface must be such that it assists the enzyme to orientate or penetrate the surface so that its active centre is in a favourable position for hydrolysis. It is suggested that electrokinetic potentials may influence other lipolytic enzymes.

Phospholipids in Fish Lipoproteins

By June Olley. (*Torry Research Station, Aberdeen*)

Four aspects of lipoproteins in fish muscle are currently being studied: the strength of bonding between lipid and protein, the nature of the lipids, the site of lipoproteins in the tissue and the post-mortem breakdown of the phospholipid moiety.

Light petroleum extracted 8% of the total lipid from cod muscle and 2% of the phospholipid. Subsequent ether extraction yielded a further 35% of the total lipid and 9·5% of the phospholipid. Freeze-dried unmacerated muscle yielded the same amount of total extractable lipid but more was available to the light petroleum; 25% of the total lipid and 8% of the phospholipid. Vandegaer, Reichmann & Cook (1956) showed that lipovitellin contained 30–50% of ether-extractable lipid. Extractability by ether may indicate weak bonding rather than free lipid. Acetone extracted 84% and 55–60% of the phospholipid in wet and freeze-dried cod muscle respectively. Lucas, Patterson & Ridout (1958) have shown that muscle phospholipids are particularly susceptible to acetone extraction.

Silicic acid column and paper chromatography have shown the muscle phospholipids of various marine Teleosts and Elasmobranchs to be qualitatively similar. Phosphophatidyl-choline and -ethanolamine are the main constituents with small quantities of phosphatidyl-serine and -inositol, polyglycerophosphatide and sphingomyelin. D. Young (unpublished results) found that the non-polar unidentified lipid with a high nitrogen content present in an ethanol–ether extract of both cod

and haddock muscle (Olley, 1956) would appear to have been an artifact of the type described by Wren (1960), probably caused by bulk preparation.

The distribution of complex lipids in non-fatty muscle is not known. Young (unpublished results) has evidence that 50–60 % of the lipid is genuinely in the myofibrils and the remainder in the lipid-rich granules. Cytochemical and histological checks for contamination of the myofibrils by aggregated mitochondria or endoplasmic reticulum (Hendler, 1956) have not been completed. Strong lipid staining has been noted in the I bands of striated muscle (Berg, 1951; Nachmais & Padykula, 1958).

Phospholipids in cod muscle are degraded *post mortem* with loss of both fatty acids at the rate of about 33 mg./100 g./day at 20°; the rate at −7° is similar. Hydrolysis in frozen muscle is most rapid between −3·3° and −4·7°. There is no initial lag in phospholipid hydrolysis in frozen cod muscle, such as that of about 10 days at 0° (Olley & Lovern, 1960). Freezing and thawing in themselves do not activate the enzyme system.

The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research.

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Phospholipids in Ascites Tumour Cells

By G. M. Gray. (*The Lister Institute, Chelsea Bridge Road, London, S.W.* 1)

The phospholipid composition of Ehrlich ascites carcinoma cells has been described by Wallach, Soderberg & Bricker (1960) with special reference to intracellular distribution. In this work the total lipid composition of Landschutz ascites carcinoma cells was studied with particular emphasis on the distribution of the phospholipids, the composition of their fatty acids and aldehydes, and the possible presence of unusual minor components. Previous study of the phospholipid composition of a variety of normal tissues (Gray, 1960; Gray & Macfarlane, 1961) formed a basis for comparison.

Freeze-dried Landschutz ascites cells (100 g.) were reconstituted with water (1:9, w/v) and the wet mass was extracted with chloroform–methanol (1:1, v/v; 2 vol.) followed by chloroform–methanol (2:1, v/v; 1 vol., two extractions). The chloroform phase of the total extract was washed three times with 0·1 M-KCl (0·2 vol.) and an interfacial layer of lipoprotein which separated at this stage was removed and the lipid content extracted and analysed separately. The main lipid extract was taken to dryness under vacuum and the phospholipids were separated from the neutral fat, cholesterol, etc. by dialysis through a rubber membrane. The phospholipids (6·6 g./100 g. dry wt. of cells) and the other lipids (7·0 g./100 g. dry wt.) were fractionated on different columns of silicic acid with the appropriate solvent systems. The computed phospholipid composition was as follows (as % of total P), cardiolipin, 2; phosphatidic acid, 2; phosphatidylethanolamine, 17; phosphatidylserine, 6; phosphatidylinositol, 6; ethanolamine plasmalogen, 5; serine plasmalogen, 1; choline plasmalogen, 6; phosphatidylcholine, 40; sphingomyelin, 12; lysophosphatidylcholine, 2. The lipoprotein (1·6 g./100 dry wt. of cells, 65 % protein) had a similar distribution of phospholipids.

The distribution of fatty acids in cardiolipin, phosphatidic acid, kephalin, kephalin plasmalogen, phosphatidylcholine and choline plasmalogen showed far less variation from class to class than those present in the normal tissues examined so far, especially with respect to the proportions of stearic, palmitic, oleic and linoleic acids. Stearic acid was the predominating saturated acid in all the phospholipids, thus differing from normal tissues in which kephalin and kephalin plasmalogen have stearic acid predominating but phosphatidylcholine and choline plasmalogen have palmitic acid as the major saturated acid.

Galactose and glucose were identified in the hydrolysis products of the kephalin fractions but no trace of sphingosine was found, thus the presence of cerebrosides and/or cytolipin H, a lipid hapten isolated from phospholipid fractions of some human tumour tissues by Rapport, Graf, Skipski & Alonzo (1959), was excluded. The carbohydrates appeared to be components of a phospholipid containing ethanolamine, the amino group of which was combined with an unknown component, possibly a fatty acid.

The presence of malignolipin in the tumour cells (Kosaki, Ikeda, Kotani, Nakagana & Saka, 1958) could not be confirmed (cf. Kogl, Smak, Veerkamp & van Deenen, 1960).

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The Phosphatidylglycerol and Sulpholipid of Plants: Asymmetry of the Glycerol Moiety

By A. A. BENSON* and M. MIYANO† (introduced by MARJORIE G. MACFARLANE). (*Department of Agricultural and Biological Chemistry, Pennsylvania State University, University Park, Pennsylvania, U.S.A.*)

The major anionic lipids of chloroplasts and photosynthetic tissues are phosphatidylglycerol and the sulpholipid, 6-sulpho-α-D-quinovopyranosyl-(1 → 1′)-2′,3′-diacylglycerol. Each exhibits a remarkably active metabolism and is labelled within minutes during photosynthesis in $^{14}CO_2$, $H_2{}^{32}PO_4^-$, or $^{35}SO_4^{2-}$. The former is also widely distributed in animal tissues where its concentration is low and often exceeded by that of diphosphatidylglycerol (cardiolipin). The deacylated derivative of each occurs in the free state in plant tissues. They are intimately involved in carbohydrate metabolism.

The plant sulpholipid differs in structure from the predominant lipids of green tissues, the galactosyl diglycerides, in that it has an α-D-glucoside structure while the galactolipids are β-linked to diglycerides. An interesting relationship between these glycolipids was revealed by the observation (Shibyua & Benson, unpublished results) that the glycosidic linkages are cleaved by β-galactosidase, normally a very specific enzyme.

The mode of phytosynthesis of the plant lipids determines the nature of the diglyceride moiety which each possesses. Acylation of the naturally occurring D-glycerol 1-phosphate results in asymmetric diglyceride derivatives. Phosphatidylation of glycerophosphate by reactions studied in liver by Kiyasu, Paulus & Kennedy (1960) would be anticipated to yield 1-phosphatidyl-L-glycerol. The reaction of cytidine diphosphate glycerol (Baddiley, Buchanan, Carss, Mathias & Sanderson, 1956) with a diglyceride would be expected to yield 1-phosphatidyl-D-glycerol. Similarly, sulphoquinovosylation of a racemic diglyceride by a nucleoside diphosphate sulphoquinovose (Yagi & Benson, unpublished results) should form a 1-sulphoquinovosyl-DL-glycerol derivative. We have examined the configuration of the glycerol moieties in these lipids.

The ^{14}C-labelled deacylated derivatives of phosphatidylglycerol, the sulpholipid, and of the galactosyldiglycerides were oxidized to glyceric acid derivatives with NO_2. The chromatographically purified glyceric acids were co-crystallized with salts of the authentic asymmetric acids. Retention of specific activity indicated identity. Each of the glycolipids yielded [^{14}C]-L-glyceric acid, thereby indicating that they are 1-glycosyl-D-glycerol derivatives. Diglycerophosphate, derived from phosphatidylglycerol by deacylation, gave [^{14}C]-DL-glyceric acid. Its phytosynthesis, therefore, proceeds by a sequence like that of Kiyasu *et al.* (1960) in which cytidine diphosphate diglyceride phosphatidylates a D-glycerol 1-phosphate, followed by removal of the phosphate monoester.

These results suggest that the rapid metabolic exchange of glycosyl and phosphatidyl groups (Ferrari & Benson, 1961) involves strictly asymmetric processes and, further, requires a pool of asymmetric diglyceride (2,3-diacyl-D-glycerol). If, as is reasonable, the third acylation of glycerol occurs in a domain isolated from the fatty acid pools involved in diglyceride formation, the product would be a spectrum of asymmetric triglycerides. One may infer that there may be unforeseen asymmetry of triglycerides in Nature.

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Mycobacterial Inositophospholipids

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Anderson, Lothrop & Creighton (1938) found inositol and mannose in mycobacterial phospholipids and Bloch (1936*a*, *b*) and Pangborn (1949) obtained nitrogen-free phospholipid fractions from *Mycobacteria*.

In previous work from the author's laboratory, nitrogen containing phospholipids from various strains of *Mycobacteria* were obtained, ornithine being the principal nitrogenous constituent (Gendre & Lederer, 1955); it has been found, more recently, that ornithine is not linked to the phospholipid, but seems to be present as an impurity in a liposoluble form.

Nitrogen-free phospholipids have now been prepared in fair yield from several strains of *Mycobacteria* and have been shown to be mixtures of phosphatidic acid (Vilkas & Lederer, 1956) phosphatidylinositol, phosphatidylinosito-mono-, -di-

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and -tri-mannosides as well as inositopentaglucosides (Vilkas, 1960; Vilkas & Lederer, 1960).

Phosphatidylinositopentamannosides of *Mycobacteria* have been described by Pangborn (1958) and Nojima (1959).

The structure of the phosphatidylinositodimannoside, which is the principal component of this mixture, has been studied and the presence of a 6-*O*-α-D-mannopyranosyl-α-D-mannopyranose linked to inositol has been proven.

Mycobacterial phospholipids are antigenic (Takahashi, Fujita & Sasaki, 1961); the possibility of immunizing animals against tuberculosis with some of these preparations was discussed.

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Studies on Plasmalogens in Nervous Tissues

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Previous investigations have established that white matter of the central nervous system contains considerably greater amounts of plasmalogens than do regions consisting predominantly of grey matter (Stammler, Stammler & Debuch, 1954). The plasmalogens in lipids isolated from whole brain have further been shown to be mainly of the ethanolamine type and to contain only small proportions of serine- or choline-containing plasmalogens (Klenk, Debuch & Daun, 1953; Ansell & Norman, 1956). In a quantitative survey of the regional distribution of ethanolamine- and choline-containing plasmalogens in the nervous system of man and animals the preponderance of the ethanolamine type was confirmed in all regions in the species examined, and was uniformly accompanied by only very low levels of choline plasmalogen (Webster, 1960).

Examination of kephalin fractions of nervous tissues, isolated by fractionation of the lipids on alumina columns, showed that these consisted of 90% or more ethanolamine phosphatide, and, in conformity with the finding by Long & Staples (1961), very little serine phosphatide. In kephalin fractions from white matter the ethanolamine phosphatide was almost entirely in the plasmalogen form; that from human-brain white matter, for example, had molar ratios of phosphorus:ethanolamine:plasmalogen:serine of 1:0·90:0·91:0·01, and was isolated in a yield of about 30 μmoles/g. of fresh tissue. Kephalin fractions from grey matter were found to be 50% in the plasmalogen form. These findings underline the suggestion by Brante (1949) that ethanolamine phosphatide in the myelin sheaths is probably all or nearly all present as plasmalogen.

The biological significance of the plasmalogens is still uncertain. One suggestion, of interest pathologically in relation to neurological disease, is that under some circumstances the aldehydogenic moiety might be split off with the consequent production of lysophosphatides (Debuch, 1957), which are known to be capable of causing potentially damaging effects on tissues (Webster, 1961).

Preliminary experiments have so far failed to indicate that the aldehyde linkage would be unstable under conditions likely to occur physiologically, or possibly in pathological tissues; the lipid appeared to be stable during incubation of plasmalogen preparations or tissue homogenates in aqueous media at 37°, so long as the pH was above 4·5.

No evidence has been found in the present experiments of enzymic hydrolysis of the aldehyde linkage of plasmalogens by rat-tissue preparations.

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Phosphatides and Active Transport with Particular Reference to the Role of Phosphatidic Acid in Sodium Transport

By L. E. HOKIN and MABEL R. HOKIN. (*Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin, U.S.A.*)

Stimulation of the secretion of water-soluble substances ranging in size from sodium chloride to proteins in a variety of exocrine and endocrine glands

is accompanied by a characteristic stimulation in the metabolism of certain phosphatides (see review by Hokin & Hokin, 1962*b*). Those phosphatides most affected are phosphatidic acid and phosphoinositide, although phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine are affected to a lesser extent in some tissues. An inositol-containing 'phosphatido-peptide' fraction is also stimulated (Huggins, 1959). In salt-gland slices the phospholipid effect occurs in secretory membranes (Hokin & Hokin, 1960) and in the pancreas it occurs in the endoplasmic reticulum (Hokin & Hokin, 1962*a*) which probably serves a secretory function (see Palade, 1962).

Several lines of evidence point strongly to the conclusion that sodium transport is coupled to the renewal of phosphate in phosphatidic acid, a process which appears to be catalysed by the cyclic action of diglyceride kinase and phosphatidic acid phosphatase, and which has been termed the phosphatidic acid cycle (Hokin & Hokin, 1962*b*). Stimulation of salt-gland slices with acetylcholine, which is the physiological stimulus of secretion in this gland, leads to about a 15-fold increase in the level of ^{32}P-labelled phosphatidic acid (Hokin & Hokin, 1960). In brain microsomal membranes, where a stimulation of the renewal of phosphate in phosphatidic acid also occurs in response to acetylcholine, diglyceride kinase and phosphatidic acid phosphatase appear to be the enzymes which catalyse this stimulation (Hokin & Hokin, 1959*a*, *b*). Kinetic studies in salt-gland slices indicate that the phosphate in the responsive phosphatidic acid molecules turns over at very rapid rates and does not mix with the phosphate of other phosphatidic acid molecules (Hokin & Hokin, 1961*b*). Ouabain, which has been shown specifically to inhibit transport of Na^+ and K^+ in a variety of tissues, elevates the level of radioactive phosphatidic acid in brain-cortex slices (Yoshida, Nukada & Fujisawa, 1961; Hokin & Hokin, 1962*b*) and in salt-gland slices (Hokin & Hokin, 1962*b*). This is compatible with the blocking by ouabain of the breakdown of phosphatidic acid, although when red-cell membranes are incubated with exogenous substrate ouabain does not appear directly to inhibit phosphatidic acid phosphatase. The net reaction of the diglyceride kinase and phosphatidic acid phosphatase reactions is an adenosine triphosphatase, and it has been found that these two enzymes have certain features is common (Hokin & Hokin, 1961*a*, 1962*b*) with the membrane $Na^+ + K^+$ adenosine triphosphatase, shown by Skou (1957) and Post, Merrit, Kinsolving & Albright (1960) to be related to transport of Na^+ and K^+.

The stimulation of phosphoinositide turnover appears to be independent of that of phosphatidic acid (Hokin & Hokin, 1960). However, the enzymes concerned and the role played by phosphoinositide in the overall secretory process are not known. Whether the small stimulations of turnover of the other phosphatides in some tissues plays a physiological role or is a secondary effect is also not known.

Possible mechanisms of coupling of active transport to phosphatide turnover have been recently discussed (Hokin & Hokin, 1960, 1962*b*).

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COMMUNICATIONS

Action of Phospholipases from *Clostridium welchii* and *Bacillus cereus* on Red-cell Membranes

By J. DE GIER, G. H. DE HAAS and L. L. M. VAN DEENEN (introduced by MARJORIE G. MACFARLANE). (*Laboratory of Organic Chemistry, State University of Utrecht, The Netherlands*)

After the discovery by Macfarlane & Knight (1941) that the haemolytic factor of *Clostridium welchii* α-toxin converted lecithins into phosphorylcholine and diglycerides, it was established that sphingomyelins are hydrolysed at a considerably lower rate, whereas isolated kephalins and synthetic phosphatidylethanolamines appeared not to be susceptible at all (Zamecnik, Brewster & Lipmann, 1947; Macfarlane, 1948; van Deenen, de Haas, Heemskerk & Meduski, 1961). The action of this enzyme has been investigated in red cells of different species of mammals (man, rat, rabbit, pig,

ox), which are known to differ greatly with respect to their phosphatide composition. After incubation with *C. welchii* toxin the red-cell ghosts were collected and determinations were carried out of the content of total lipids and phosphatides as well as of the individual classes of phosphatides (de Gier & van Deenen, 1961). All major types of phosphatides, namely lecithins, sphingomyelins and, surprisingly, also kephalins, appeared to be hydrolysed, the rates of breakdown being different not only between the various classes of phosphatides, but for a certain type of phosphatide also between the red cells of some animal species.

The discrepancy with respect to the resistance of pure phosphatidylethanolamines to the enzymic action and the apparent degradation of these kephalins when present in red-cell membranes or in brain thromboplastin (Kushner & Feldmann, 1958), was abolished by studying the action of the enzyme on synthetic phosphatidylethanolamines emulsified together with synthetic lecithins. In spite of the fact that the synthetic kephalins alone, being well emulsified, were not degraded, it appeared that in the presence of choline-containing phosphatides the phosphatidylethanolamines were actually attacked by *C. welchii* phospholipase, thereby yielding phosphorylethanolamine. Probably these conditions favour the requirements in charge and orientation of the molecules necessary for an enzyme–substrate interaction. In this respect it is worth noting that recently R. M. C. Dawson & A. D. Bangham (personal communication) and Matsumoto (1961) observed a breakdown of isolated phosphatidylethanolamines by *C. welchii* α-toxin.

Contrary to *C. welchii* phospholipase, *Bacillus cereus* phospholipase was found, in agreement with Chu (1949), to act readily on synthetic phosphatidylethanolamines, also without the addition of choline-containing phosphatides. Consequently the latter enzyme attacked more readily kephalins in red-cell membranes than did the *C. welchii* phospholipase. Since the mode of action of both bacterial phospholipases appeared to be identical, namely, the releasing of the phosphoryl nitrogenous moiety from the phosphatide molecule, both enzymes probably differ with respect to their distribution of charge.

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Synthetic Mixed-acid Kephalins; Hydrolysis by 'Phospholipase A' and Some Properties of Their Sodium Salts

By G. H. DE HAAS and L. L. M. VAN DEENEN (introduced by MARJORIE G. MACFARLANE). (*Laboratory of Organic Chemistry, State University of Utrecht, The Netherlands*)

Extending the synthesis of mixed-acid lecithins (de Haas & van Deenen, 1960*a*, *b*) the preparation was undertaken of L-α-phosphatidylethanolamines and L-α-phosphatidylserines, containing two different fatty acid constituents. Two fully synthetic methods were developed, allowing the preparation of kephalins containing either two saturated fatty acids of unequal chain length or one saturated and one unsaturated fatty acid in different positions (de Haas & van Deenen, 1961*a*, *b*; Daemen, de Haas & van Deenen, to be published).

The action of 'phospholipase A' from snake venom and other origins, which enzyme is known to release one mole of fatty acid from the phosphatide molecule, has been studied in aqueous emulsions of the mixed-acid kephalins. In order to establish the site of the enzymic action, adequate samples of the incubation mixture were subjected to chromatography on silica-impregnated paper (Marinetti, Erbland & Kochen, 1957). The separated spots of the formed lyso compound, the intact phosphatide eventually remaining, and the liberated fatty acid, were directly treated with methanol–hydrochloric acid. The fatty acid methyl esters thus obtained were analysed by means of gas–liquid chromatography. In all cases studied only the fatty acid present in β-ester position of the phosphatidylethanolamines and phosphatidylserines appeared to be released by the different enzyme preparations used, while the lyso compounds formed always contained exclusively the γ-fatty acid. These results are in keeping with the findings on synthetic mixed-acid lecithins, as well as with the mode of action of the enzymes in ethereal medium (de Haas, Mulder & van Deenen, 1960; de Haas & van Deenen, 1961*c*; cf. also Tattrie, 1959; Hanahan, Brockerhoff & Barron, 1960). In addition, fresh information was obtained with respect to differences in the rate of hydrolysis of distinct types of phosphatides and on the susceptibility of structurally related compounds, e.g. glycol phosphatides.

In connexion with an investigation into the possible functions of acidic phosphatides in the active

transport of ions across red-cell membranes, the chromatographic behaviour of ^{22}Na-labelled salts of phosphatidylethanolamine and phosphatidylserine was studied, and a comparison has been made between the infrared spectra of the salts and the metal-free form of both types of phosphatides.

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Incorporation of Inorganic Phosphate into Brain Phosphoinositides

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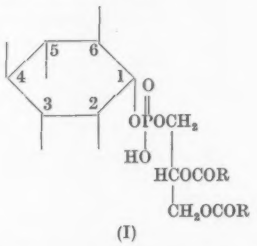
We have presented evidence that brain phosphoinositide is a complex of three compounds, 1-phosphatidyl-L-myoinositol (I), 1-phosphatidyl-L-myoinositol 4-phosphate (II), and 1-phosphatidyl-L-myoinositol 4:5-diphosphate (III) (Grado & Ballou, 1960, 1961; Tomlinson & Ballou, 1961; Brockerhoff & Ballou, 1961).



(I)

The close structural relationship of these compounds suggests that II and III arise from I by a stepwise phosphorylation. As a test of this hypothesis, we have studied the incorporation of inorganic ^{32}P into the phosphoinositides of rabbit-brain slices. After a 2 hr. incubation of 2 g. of brain slices in 50 ml. of Krebs–Henseleit solution containing 1 mc of inorganic ^{32}P, the phospholipids were extracted from the tissue, deacylated according to Dawson (1954), and the products corresponding to I, II and III were separated by a combination of ion-exchange and filter-paper chromatography. About 0·1% of the radioactivity in the solution was incorporated into the total phospholipids, and, of this, 50% was in the inositide complex.

The total ^{32}P in I, II and III, as well as the fraction in diester form on position 1 and in monoester form on positions 4 and 5, were determined as by Brockerhoff & Ballou, 1961. The results were as follows (counts/min./μmole): total activity; I, 73 500; II, 194 000; III, 213 000; activity in 1-position; I, 73 500; II, 17 500; III, 3000; activity in monoester form; I, none; II, 176 500; III, 210 000. The results reveal that there is a rapid labelling of the phosphate monoester groups in II and III, and a much slower incorporation of ^{32}P into the phosphatidyl phosphate group. The activity in the latter decreases markedly in going from I to II to III. Both results are consistent with a mechanism for the biosynthesis of the brain inositide complex that involves the stepwise phosphorylation of I→II→III. The rapid turnover of the monoesterified phosphate groups in the brain inositide complex might also reflect a unique aspect of their metabolic function.

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On the Aldehydes of Plasmalogens of Different Kinds of Mammalian Brain

By Hildegaard Debuch (introduced by Marjorie G. Macfarlane). (*Physiological-Chemisches Institut der Universität, Köln, Germany*)

Supplementing the work on plasmalogens of brain (Klenk & Debuch, 1954; Debuch, 1956, 1958, 1959) the composition of the total aldehyde mixture from different mammalian brains has been investigated.

Brains from man, ox, horse, pig, rabbit and rat were homogenized, dried with acetone and extracted as previously described (Debuch, 1956). The glycerophospholipids of the acetone, ether, and chloroform–methanol (1:3, v/v) extracts were

isolated by precipitation. The aldehydes of the plasmalogens were converted by methanolysis into the dimethylacetals, which were separated from the fatty acid methyl esters after saponification with methanolic sodium hydroxide. Sometimes saponification must be repeated to obtain pure dimethylacetals, checked by plasmal estimation according to Feulgen, Boguth & Andresen (1951). The quantitative composition of the aldehydes was estimated by gas chromatography on a succinic acid–ethanediol polyester column.

The composition of the dimethylacetals from the mammalian brains mentioned above was very similar. Palmitic, stearic and octadecenoic aldehydes were the only major components, with trace amounts of two or three other aldehydes. The only difference between the aldehyde mixtures under investigation was the variation in the quantity of these three aldehydes. This result seems to be remarkable in comparison with the aldehydes from other sources, as recently reported by Gray (1960).

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A Study of the Plasma Fatty Acids in Xanthomatosis By Gas–Liquid Chromatography

By J. D. Billimoria. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

The individual plasma fatty acids of a small group of five normal subjects and six subjects with xanthomatosis were compared by gas–liquid chromatography. The fatty acids derived from total plasma lipids showed somewhat lower levels of polyunsaturated acids in xanthomatosis, the remaining fatty acids being similar in the two groups.

The plasma lipids were separated into cholesterol esters, triglycerides and phospholipids by silicic acid chromatography and the fatty acids of each lipid fraction were separately analysed. The principal differences were found in the cholesterol ester fractions; three subjects with idiopathic hyperlipaemia showed considerably lower levels of linoleic acid and correspondingly higher levels of palmitic, stearic and oleic acids. The two other fractions showed only minor changes in their fatty acid contents. In the other three subjects with xanthomatosis and normal triglyceride levels, these changes were not observed.

The study of a larger number of subjects is now in progress.

I wish to thank Dr E. M. Jepson for helpful discussion and specimens of xanthomatosis serum.

The Probable Occurrence of Long-Chain Ether Analogues of Phosphatidylethanolamine in Brain Tissue

By G. B. Ansell and Sheila Spanner. (*Department of Experimental Psychiatry, The Medical School, Birmingham* 15)

When brain phospholipids are saponified with dilute alkali at 37° and then treated with dilute acid, the remaining lipid-bound phosphorus can be further hydrolysed with 2N-methanolic hydrochloric acid to yield a number of water-soluble phosphate esters (Dawson, 1960; Ansell & Spanner, 1961). Hydrolysis products of sphingomyelin account for the greater part of the fraction whilst another ester has been identified as α-glycerophosphate (Davenport & Dawson, personal communication; Ansell & Spanner, 1961); this glycerophosphate presumably derives from a cyclic acetal of a plasmalogen formed during the saponification and subsequent acidification (Davenport & Dawson, 1961).

A proportion (20–27 %) of the acid- and alkali-stable phospholipid, however, remained chloroform- and ether-soluble after the hydrolysis with methanolic hydrochloric acid (Ansell & Spanner, 1961). The stability of the lipid and its positive ninhydrin reaction suggested that it might be a partial hydrolysis product of a long-chain glycerol ether analogue of phosphatidylethanolamine. Lipids of this nature have been identified in egg yolk by Carter, Smith & Jones (1958) and in brain by Svennerholm & Thorin (1960).

Through the generosity of Dr T. H. Bevan samples of synthetic 2-aminoethyl 2-*O*-stearoyl-batyl (and -chimyl) phosphates (Baylis, Bevan & Malkin, 1958) were made available to us. When these were saponified and hydrolysed with 2N-methanolic hydrochloric acid it was found that most of the phosphorus remained chloroform-soluble. Chromatography of this hydrolysis product on silicic acid columns using stepwise dilutions of chloroform with methanol showed that it could be eluted with a 4:1 (v/v) mixture of chloroform and methanol. The material obtained from brain tissue behaved in an analogous fashion and the eluted lipid moved in

each instance as a single ninhydrin-reacting component on silica-impregnated papers in di-*n*-butyl ether–acetic acid–chloroform–water (40:35:6:5, by vol.) (Marinetti & Stotz, 1956). Isotope-dilution experiments performed with synthetic lipid and lipid from the brains of rats injected with ^{32}P, also indicated the similarity between the two substances.

Prolonged hydrolysis of both synthetic and brain material in 2N-hydrochloric acid at 123° yielded a water-soluble ninhydrin-reacting compound in addition to ethanolamine and inorganic phosphate. The chloroform-soluble, phosphorus-free material obtained as a result of this hydrolysis yielded formaldehyde on periodate oxidation, as would be expected from long-chain α-glycerol ethers, but the stoicheiometric relationships were difficult to determine for the brain lipid because of contamination with sphingosine, which presumably derived from the cerebrosides.

In rat brain this ethanolamine lipid accounted for only 1·8% of the total lipid phosphorus in 8-day-old rats and 3·1% in the adult. Its incorporation of [^{32}P]orthophosphate *in vivo* proceeded at a far greater rate than that of sphingomyelin.

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Incorporation of [^{32}P]Orthophosphate into the Phosphate Esters of Skin Maintained *in vitro* in the Absence of Added Substrate

By H. J. YARDLEY and G. GODFREY (introduced by G. B. ANSELL). (*Medical Research Council Unit for Research on the Experimental Pathology of the Skin, University of Birmingham*)

It has been shown that, in the absence of added substrate, skin can respire *in vitro* at near normal rates for up to 4 hr. (Cruickshank, Trotter & Cooper, 1957). More recently, Cruickshank, Trotter & Cooper (unpublished results) have shown that utilization of endogenous glucose, glycogen and protein cannot account for more than 40% of this respiration. As phosphorus metabolism is so closely linked with energy-producing reactions, the uptake of [^{32}P]orthophosphate into the phosphate esters of skin, maintained in the presence and absence of glucose, was examined using a method based on that described by Brooks, Lawrence & Ricketts (1959).

Slices of guinea-pig-ear skin were incubated for 4 hr. at 37° on Krebs–Ringer phosphate (Krebs, 1933) containing [^{32}P]orthophosphate with or without the addition of glucose. The skin was then frozen in liquid nitrogen, extracted with 10% (w/v) trichloroacetic acid at 0°, chromatographed in two dimensions and radioautographs prepared.

Inspection of the radioautographs revealed much less labelling of glycolytic intermediates in skin maintained in the absence of added substrate compared with skin maintained on a medium containing glucose. Of more interest was the increased activity of a single spot on chromatograms of extracts derived from skin maintained on glucose-free medium. This spot ['spot 26' of Brooks *et al.* (1959)] was separated into two compounds by further chromatography in solvents containing phenol. The fast-moving compound in these solvents was identified as phosphorylcholine by co-chromatography with an authentic sample and by measurement of its rate of hydrolysis in N-HCl at 100°. The increased activity in 'spot 26' was almost entirely due to the greatly increased amount of labelled phosphorylcholine.

The formation of ^{32}P-labelled phosphorylcholine in skin maintained in the absence of added substrate is put forward as evidence that skin can derive energy from catabolism of its phospholipids according to the scheme proposed by Dawson (1956). The liberated fatty acids can be made to yield energy by β-oxidation and incorporation into the Krebs cycle, while choline and glycerol become available for phosphorylation by ^{32}P-labelled ATP.

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Automatic Analysis of Iodoamino Acids in Digests of Iodinated Proteins

By RICHARD J. BLOCK and RICHARD H. MANDL. (*Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, U.S.A.*)

The amount of hormonal activity in iodinated proteins can only be ascertained with relative accuracy

by biological assay (Kroc, Phillips, Stasilli & Malament, 1954). Chemical assay (Blau, 1935) is subject to considerable error because of the presence of tri-iodothyronine in the thyroxine fraction. Mandl & Block (1959) published a method of estimating the iodoamino acids in thyroid digests based on the separation of iodide, di-iodotyrosine, monoiodotyrosine, thyroxine and tri-iodothyronine by paper chromatography. This procedure, although useful, requires meticulous attention to details and is exceedingly time-consuming.

The technique described in this paper permits the quantitative determination of iodide, di-iodotyrosine, monoiodotyrosine, thyroxine and tri-iodothyronine in enzymic digests of less than 1 mg. of iodoproteins by a fully automatic method. Unidentified iodocompounds in the digests are also revealed.

Digestion of iodoproteins. Approximately 10 mg. of the sample are suspended in 2·5 ml. of 10 % (v/v) ethanolic 0·01 M-$(NH_4)_2CO_3$ buffer, pH 8·4, and 0·1 mg. of enzyme P from *Streptomyces griseus* in 0·5 ml. of ethanolic $(NH_4)_2CO_3$ is added. After 48 hr. at 40°, 1 ml. of ethanol is added and the volume is brought to 10 ml. with 0·2 M-$H{\cdot}CO_2NH_4$–$H{\cdot}CO_2H$ buffer, pH 3·9, in 30 % (v/v) ethanol.

Column chromatography. Dowex 50 W (X-4, 20–40 μ beads) is used to prepare a 0·9 × 15·0 cm. column. The resin is converted into the NH_4^+ cycle and then buffered with 0·2 M-$H{\cdot}CO_2NH_4$–$H{\cdot}CO_2H$ in 30 % (v/v) ethanol at pH 3·9 (Lerner, 1960). A portion of the iodoprotein (200–1000 μg.) is introduced into the column. The iodoamino acids are separated by gradient elution (Peterson & Sober, 1960). Chamber no. 1 contains 40 ml. of 0·2 M-$H{\cdot}CO_2NH_4$–$H{\cdot}CO_2H$, pH 3·9, in 30 % (v/v) ethanol; no. 2 contains 20 ml. of ethanolic 0·2 M-$H{\cdot}CO_2NH_4$ buffer, pH 3·9, plus 20 ml. of 0·2 N-NH_4OH in 30 % (v/v) ethanol; no. 3 contains 40 ml. of 30 % (v/v) ethanol; no. 4 contains 40 ml. of ethanolic 0·5 M-NH_4OH. The flow rate is 0·6ml./min. The effluent is continuously mixed with 2·0 % (w/v) $Ce(HSO_4)_4$ in 3 N-H_2SO_4, 2 N-H_2SO_4 and 0·3 % $NaAsO_2$ in water by means of the AutoAnalyzer. The mixture is heated at 60° for approximately 10 min. and the degree of fading is measured by means of a flow colorimeter at 420 mμ. Iodide, di-iodotyrosine, monoiodotyrosine, thyroxine and tri-iodothyroxine are determined in 5 hr. with a reproducibility of ±6 %. At least one unidentified iodine-containing compound is present in appreciable quantities in thyroid digests.

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The Effect of Inhibitors on the Activities of Lactic Dehydrogenase Preparations

By D. T. Plummer and J. H. Wilkinson. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

Although the reversible reduction of pyruvate is catalysed by all mammalian tissue lactic dehydrogenases, certain physicochemical properties vary according to the source of the enzyme. Differences in electrophoretic mobility, pH optima and apparent Michaelis constants have been observed by Plagemann, Gregory & Wróblewski (1960*a*, *b*) and Nisselbaum & Bodansky (1959) have reported that different tissue enzymes have distinct serological properties.

During studies of the substrate specificities of a series of lactic dehydrogenase preparations, Meister (1950) found that greatest activity was exerted against pyruvate and α-oxobutyrate, and the relative activities against these substrates have recently been shown to be characteristic of the tissue of origin (Elliott & Wilkinson, 1961; Wilkinson, Cooke, Elliott & Plummer, 1961).

Novoa, Winer, Glaid & Schwert (1959) observed that with pyruvate as substrate crystalline ox-heart lactic dehydrogenase is competitively inhibited by oxamate but non-competitively by oxalate. The effects of these inhibitors on the activities exerted against pyruvate and α-oxobutyrate by purified ox-heart and rabbit-muscle lactic dehydrogenases and by crude extracts of various human tissues have now been investigated.

In all cases oxamate had a greater effect when α-oxobutyrate was used as substrate than when pyruvate was employed. This is in accord with the finding that the affinity of lactic dehydrogenases for α-oxobutyrate is less than that for pyruvate in all the tissues studied by Wilkinson *et al.* (1961), and also with the competitive nature of the inhibition exerted by oxamate.

Activity against both substrates was inhibited equally by oxalate, and with a given enzyme preparation identical inhibitor constants were obtained irrespective of the substrate employed. Enzyme preparations from different tissues, however, gave different values.

The effects of oxamate is therefore to distinguish between the two substrates and that of oxalate to

differentiate between the different sources of the enzyme. In the case of the human-tissue preparations, the reciprocal inhibitor constant for oxalate decreased linearly with the relative activities against the two substrates. Thus the affinity for oxalate may also be used as a means of distinguishing the tissue of origin.

The findings are consistent with the view that pyruvate and α-oxobutyrate form enzyme–substrate complexes at similar active centres on lactic dehydrogenases from different tissues and that these enzymes owe their individual properties to variations in their peptide chains.

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The Localization of Transaminases and Dehydrogenases in Agar Gels after Electrophoresis

By J. W. Boyd. (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

Several methods have been used to locate dehydrogenases in starch and agar after electrophoresis. Wieland & Pfleiderer (1957) demonstrated bands of lactate dehydrogenase activity on paper and starch. The strips were treated with a solution of pyruvate and DPNH, and viewed in ultraviolet light. As the fluorescent DPNH was oxidized by the enzyme dark bands appeared. Markert & Møller (1959) devised an indirect method for locating dehydrogenases in starch gels after electrophoresis. DPN or TPN reduction is linked to a neotetrazolium system so that a purple formazan dye is precipitated in regions of dehydrogenase activity. Wieme (1959) located lactate dehydrogenase in agar gels by scanning at 340 mμ in a spectrophotometer.

The new method proposed gives a photographic record of the enzyme bands and requires only an ultraviolet lamp and contact paper. Following electrophoresis the agar gel is overlaid with agar containing DPNH and substrates and incubated for 30–60 min. at room temperature. In a darkroom contact paper is placed under the gel, exposed to ultraviolet light and developed. With correct exposure times light only penetrates the gel where DPNH has been oxidized by enzyme action, so enzyme bands appear black on a white background. Using this method glutamate–oxaloacetate transaminase, and lactate and malate dehydrogenases have been located in agar gels after electrophoresis of various tissue extracts.

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Two New Families with Bisalbuminaemia: An Examination of Possible Links with other Genetically Controlled Variants

By K. B. Cooke, T. E. Cleghorn and Eunice Lockey. (*Westminster Medical School, Horseferry Road, London, S.W. 1; South London Blood Transfusion Centre and Royal Masonic Hospital*)

Knedel (1957) reported the occurrence of two electrophoretically distinct albumins in the plasma of the members of one family and showed that this characteristic was inherited. The pedigrees of a further seven families have since been reported. It is now generally accepted that albumin synthesis is controlled by two co-dominant genes, bisalbuminaemia occurring in the heterozygote.

The pedigree of two further apparently unrelated families, A.D.M. and E.D. with the slow form (Wieme, 1961) of bisalbuminaemia are presented. A survey has been carried out to see if the albumin genes segregate with those of any other readily detectable variant. In neither family were the members with bisalbuminaemia distinguishable from other members of the family by any obvious physical characteristic or pathological condition, nor was there a link with any other genetically controlled plasma protein, nor with phenyl thiourea tasting. As regards the blood groups, it can be stated that the albumin genes are not part of any of the common blood or saliva systems, nor are they partially sex linked.

In an attempt to find other families we have examined over 12 000 sera but without success. Further work is in progress on the isolation and

characterization of the two albumins and their possible link with other inherited conditions.

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Attempts to Detect Lycopersene in Carotenogenic Systems

By B. H. Davies, T. W. Goodwin and E. I. Mercer. (*Department of Agricultural Biochemistry, University College of Wales, Aberystwyth*)

In the biosynthesis of triterpenes, isoprene units are built up to form the C_{30} precursor, squalene, which then cyclizes (see, for example, Popják, 1958). By analogy, the C_{40} precursor of the carotenoids would be lycopersene. We have developed a method for the separation, detection and identification of small quantities of lycopersene, squalene, and related hydrocarbons, and used it in attempts to detect lycopersene in carotenogenic systems.

The hydrocarbons are separated by ascending adsorption chromatography on thin layers of Kieselgel G supported on glass plates. With light petroleum as solvent, the R_F values of squalene, lycopersene, phytoene and phytofluene are 0·41, 0·30, 0·21 and 0·12 respectively. The plates are stained with iodine vapour, and as little as 0·05 μg. of any component can be detected as a yellow or brown spot.

By this method, the presence of squalene, phytoene and phytofluene has been demonstrated in the unsaponifiable fraction of cells of *Rhodospirillum rubrum* in which normal carotenogenesis has been inhibited by diphenylamine (Goodwin & Osman, 1954). These substances do not occur in the normally pigmented bacteria. Squalene is also present in etiolated maize seedings (and, to a lesser extent, in green seedlings), carrot root and ripening tomatoes. Lycopersene has not been detected in any of these systems.

[2-^{14}C]Mevalonic acid, an effective terpenoid precursor, is incorporated into the hydrocarbon fraction of etiolated maize seedlings. While squalene is heavily labelled, no significant radioactivity could be detected in the 'lycopersene' fraction.

The significance of these findings in relation to the problem of carotenogenesis was discussed.

Thanks are due to Dr O. Isler (F. Hoffman–La Roche, Basle) for a sample of synthetic lycopersene, to Professor B. C. L. Weedon for a sample of natural phytoene, to the Agricultural Research Council for a studentship (B. H. D.), to the U.S. Air Force for financial support (Contract AF 61[052]-355), and to Miss A. Darlington and Mr D. Jones for technical assistance.

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Proteins of Human-Tissue Extracts: Separation by Column Chromatography

By D. Burston, M. P. Tombs and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

Attempts to find the origin of the glycoproteins which are known to accumulate in the blood of patients and animals with tumours (Lockey, Anderson & Maclagan, 1957; Campbell, Kernot & Roitt, 1959; Darcy, 1960; Tombs, James & Maclagan, 1961) have made little progress because of the difficulty of separating these proteins from others, particularly nucleoproteins, in tissue extracts. We have found that chromatography on diethylaminoethylcellulose overcomes this difficulty and have investigated a number of normal and tumour-containing organs, obtained either at autopsy or operation, by this method. Tissues were washed, homogenized, centrifuged and the supernatant proteins fractionated using a concave gradient system into six distinct, but heterogeneous, groups, called A–F in order of elution. The soluble RNA in the extract was eluted last and was well separated from the main protein components.

Although the chromatographic patterns obtained from the various tissues analysed were qualitatively similar, quantitative differences were observed. In liver, the level of the basic A peak proteins increased in the order: secondary liver tumours: tissues adjacent to tumours: normal liver. In other organs the tumour also contained less A protein than the adjacent tissue. The level of the more acidic D and E peak proteins, which contained considerable amounts of hexose, was higher in tumours and adjacent tissue than in normal tissue.

The pooled fractions were concentrated by pressure filtration and examined by agar electrophoresis. Peak A was found to consist of at least four components migrating towards the cathode, while the protein in peak D and E was chromatographically and electrophoretically similar to serum α_1-globulins. A maximum level for contamination by serum was deduced from the haemoglobin content of the extracts, and showed that such contamination was not the source of these glycoproteins.

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The Effect of Hydrolysis on the Ultraviolet Absorption of Proteins near 210 mμ

By M. P. Tombs and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, Horseferry Road, London, S.W.* 1)

Estimation of proteins by spectrophotometry at 210 mμ has proved a useful and versatile method in our hands (Tombs, Souter & Maclagan, 1959) and is thought to depend mainly upon absorption by the peptide bond at this wavelength. A recent observation, however, indicated that peptic hydrolysis failed to produce the expected fall in absorption at 210 mμ (W. H. Taylor, personal communication). Accordingly, the effect of acid and enzymic hydrolysis on the ultraviolet absorption spectra of some typical proteins has been studied in the region below 240 mμ. Cold 6N-HCl increased absorption at 210 mμ of bovine and human albumin but had little effect on that of bovine γ-globulin or protamine sulphate. Heating in 6N-HCl produced in all proteins studied a rapid drop in absorption at 210 mμ to approximately half the initial value. Papain hydrolysis of albumin produced a rise followed by a fall in the absorption at 210 mμ. Tryptic hydrolysis of protamine produced a fall in absorption without a preliminary rise. Correlation of these results with biuret tests suggested that the fall in ultraviolet absorption on hydrolysis was due to peptide bond splitting; the initial rise with albumin was ascribed to denaturation.

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Phosphomonoesterase Activity of Mouse-liver Preparations at pH 5·9

By M. W. Neil. (*Department of Biochemistry, London Hospital Medical College, Whitechapel, London, E.* 1)

Macdonald (1961) has recently investigated mouse-liver acid phosphatase using phenyl phosphate as substrate at pH 5·9. Macdonald's observations resembled those we have made on acid phosphatase in guinea-pig-liver subcellular fractions but our interpretation of the results was different. We therefore made a preliminary study of the hydrolysis of phenyl phosphate by mouse-liver preparations in an attempt to clarify the nature of the enzymes responsible.

Livers of mice of the CBA strain were homogenized in water as described by Macdonald (1961) and nuclei and cell debris were removed by centrifugation. The cytoplasmic extract (E) was subdivided into a washed agglutination (pH 5) precipitate (P) and a soluble fraction (S) (DeDuve, Berthet, Hers & Dupret, 1949). Hydrolysis of phenyl phosphate by each of these preparations was investigated after various periods of pre-incubation at approx. pH 6. The activity of E and P, but not S, fell rapidly with increasing time of pre-incubation. The hydrolysis at pH 5·9 of glucose 6-phosphate, phenyl phosphate and β-glycerophosphate by the preparations before and after approx. 2 hr. pre-incubation at 37° and pH 5·9 was also investigated. Glucose 6-phosphate was hydrolysed vigorously by E and P, but only very slowly by S, before pre-incubation. Hydrolysis by E and P after pre-incubation was found to be reduced to less than 5% of the initial rate; the rate of hydrolysis by S remained virtually unchanged. Phenyl phosphate was hydrolysed rapidly by E and P, and also by S but at a lower rate. The ratio of activities P:S was about 5:1. After preincubation this ratio was about 1·5:1 owing entirely to a reduction in the activity of P. The rate of hydrolysis of β-glycerophosphate at pH 5·9 was low in all preparations and the ratio of activities P:S was about 1·4:1 initially. After pre-incubation at pH 5·9 the activity of P fell to about 70% of the initial value while that of S remained constant. The labile component of E, but not of P, was protected by malonate (0·15M, pH 5·9) from inactivation during preincubation.

These experiments suggest that at least two enzymes are responsible for the hydrolysis of phenyl phosphate at pH 5·9 by mouse-liver homogenates. One enzyme, much the more active using this substrate, is labile and resembles glucose 6-phosphatase, while the other is stable and resembles true lysosomal acid phosphatase. Further, rat-liver glucose 6-phosphatase (Beaufay, Hers, Berthet & DeDuve, 1954) possesses many of the characteristics of the enzyme described by Macdonald (1961).

While the results reported by Macdonald (1961) may indeed represent interesting species and mouse strain differences in the properties of lysosomal acid phosphatase, it is suggested that an alternative explanation is possible.

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Electron-Paramagnetic-Resonance Studies of Copper Associated with Cytochrome Oxidase

By D. E. GRIFFITHS* and M. BEINERT. (*Institute for Enzyme Research, University of Wisconsin, Madison, Wis., U.S.A.*)

The presence of copper in cytochrome oxidase preparations has been noted by many investigators but it is only recently that the role of copper in the cytochrome oxidase reaction has been studied intensively. Evidence is accumulating which suggests that the copper associated with cytochrome oxidase has properties which would qualify it as a component oxidation–reduction catalyst of the oxidase. This paper presents work on reactions and properties of the copper of cytochrome oxidase insofar as they are amenable to study by electron-paramagnetic-resonance (EPR) spectroscopy.

The EPR spectrum of oxidized cytochrome oxidase from ox-heart mitochondria (Griffiths & Wharton, 1961*a*) is very similar to that of known copper proteins such as laccase or ceruloplasmin in which copper is present in the cupric form. The EPR spectrum of the isolated oxidase is almost identical to that shown by mitochondrial fragments (ETP_H) which contain a functioning cytochrome chain and are capable of oxidative phosphorylation and supports the view that the environment of the copper has not been modified significantly during the purification procedure. The EPR spectra leave no doubt that the copper in cytochrome oxidase is reduced to the cuprous form when the enzyme is reduced by substrate (Sands & Beinert, 1959). This has been confirmed by chemical analysis (Griffiths & Wharton, 1961*b*). Experiments in which the haem component of the oxidase was followed by optical spectroscopy simultaneous with EPR measurements on equally treated portions shows that the oxidation state of the copper closely follows that of the haem—from complete oxidation through the steady state to complete reduction and back to the oxidized form. Reduction of copper and haem both show dependence on cytochrome *c* concentration.

The EPR spectra also show that known inhibitors of cytochrome oxidase such as cyanide and hydroxylamine produce definite changes in the binding state of the copper. No effect was observed with azide. However, after addition of cyanide or azide, reduction of the copper by substrate proceeded considerably faster, and no re-oxidation could be observed on prolonged aeration. This behaviour again parallels that of the haem component of the oxidase.

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The experiments described support the view that copper has the general properties of a functional constituent of cytochrome oxidase.

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The Synthesis of Cystathionine by *Escherichia coli*

By R. J. ROWBURY. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Studies with induced mutants of *Escherichia coli* suggest that cystathionine is a precursor of methionine in this organism (Davis, 1955), but experiments on the effect of cystathionine on the incorporation of ^{35}S of sulphate or cysteine into methionine (Roberts, Cowie, Abelson, Bolton & Britten, 1955) do not support this view. Although the accumulation of cystathionine by a strain of *Neurospora crassa* requiring methionine has been reported (Horowitz, 1947), the synthesis of cystathionine by cell-free preparations of microorganisms has not been described.

The synthesis of cystathionine, or a closely related compound, has now been obtained with ultrasonic extracts of an auxotrophic strain (26/18) of *E. coli* blocked before homocysteine. The product supported the growth of *E. coli* 122/33 (an auxotroph responding to cystathionine, homocysteine, or methionine) but not of *E. coli* 26/18 (which responds only to homocysteine or methionine); when chromatographed on paper in butanol–acetic acid–water (2:1:1, by vol.) it had an R_F value almost identical to that of cystathionine.

The incubation mixture producing the active compound contained (in phosphate buffer, pH 7·8) DL-homoserine, L-cysteine, sodium succinate, CoA, glucose, ATP, extract of heated *E. coli*, and dialysed ultrasonic extract of *E. coli* 26/18. Homoserine and cysteine were suggested as precursors of cystathionine by work with induced mutants (Davis, 1955), and the formation of cystathionine was dependent on the presence of both. Absence of either or both of these amino acids reduced cystathionine formation to about 10%; aspartate replaced homoserine only partially. The synthesis of cystathionine was also dependent on succinate, the absence of

which decreased synthesis to 15 %; absence of CoA halved cystathionine production. Glucose and ATP were also necessary for optimal formation of cystathionine which decreased to 15 % in the absence of both. No requirement for pyridoxal phosphate was found even after prolonged dialysis of the extract followed by treatment with Dowex-1 resin.

By the use of mixtures of extracts of several mutants unable to synthesize cystathionine (i.e. requiring cystathionine or homocysteine or methionine for growth) evidence has been obtained that the cystathionine-forming system contains at least two enzymes. The nature of a comparatively stable intermediate formed by one of these enzymes is at present under investigation.

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A Modified Carbazole Reaction for Uronic Acids

By T. Bitter* and R. Ewins (introduced by H. Muir). (*Medical Unit, St Mary's Hospital, London, W.* 2)

In keeping with the finding by Aminoff, Morgan & Watkins (1952), that borate ion enhances the Morgan–Elson reaction, it was established by Gregory (1960) that this ion also increases the colour yield in Dische's (1947, 1955) carbazole reaction by approximately 60 % in 83 % sulphuric acid. For the modification of Dische's reaction suggested here, the reaction takes place in 87 % sulphuric acid, when the sensitivity is increased by approximately 100 %, except for heparin. The colour yield is linear between 4 and 60 μg. of uronic acid/ml. for the following substances: chondroitin sulphates A, B and C, a polysaccharide from aorta (Muir, 1961), heparin monosulphate, heparin, hyaluronate, galacturonolactone and glucuronolactone. For chromatographic assays in the presence of salt or buffers as well as quaternary ammonium compounds there is no reduction in colour yield from any of these compounds except heparin, where the optical density is depressed by 33 % in salt concentrations above 0·4 N-chloride.

The reagents are (i) 195 ml. of conc. H_2SO_4 (AnalaR) and 5 ml. of M-$Na_2B_4O_7$, $10H_2O$; (ii) 0·1 % commercial carbazole (British Drug Houses Ltd., not recrystallized from benzene) in ethanol. This reagent is stable for 3 months if kept cold in the dark.

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Sulphuric acid reagent (6 ml.) is placed in glass-stoppered test tubes, cooled to approximately 0°. The test sample (1 ml.) is added and the mixture shaken gently at first, and then vigorously. The tubes are heated in a boiling distilled-water bath for 15–20 min., and cooled in water to room temperature. The carbazole reagent (0·2 ml.) is added, the tubes shaken again, heated at 100° for a further 10 min. and kept in the dark for 3 hr. The optical density is read at 530 mμ. The pink colour appears immediately after the second heating but increases by approximately 10 % during the first 3 hr. and is stable for another 2½ hr.

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Inhibition of Cell-wall Mucopeptide Synthesis in *Escherichia coli* by Benzylpenicillin and 6-[D(−)-α-Aminophenylacetamido]penicillanic acid ('penbritin')

By H. J. Rogers and J. Mandelstam. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Lederberg (1956) observed the formation of spheroplasts in penicillin-treated *Escherichia coli* and suggested that the drug acted by preventing cell-wall formation, but most of the evidence for such a theory comes from work on staphylococci. Park (1952) showed that penicillin caused accumulation of uridine-linked peptides, believed by Park & Strominger (1957) to be precursors of wall mucopeptide. The inhibition of mucopeptide synthesis by the penicillins in washed staphylococci has been demonstrated by Mandelstam & Rogers (1959), Park (1958) and Rogers & Jeljaszewicz (1961).

A similar interpretation of benzylpenicillin action in *E. coli* has been proposed by Nathenson & Strominger (1961), but contested by Trucco & Pardee (1958), who failed to obtain inhibition of [^{14}C]glucose incorporation into the wall, by Meadow (1960), who found no inhibition of incorporation of diamino[^{14}C]pimelic acid (DAP), and by Hugo & Russell (1960*a*, *b*) who compared the lethal effects of the penicillins with the formation of spheroplasts in the presence of the antibiotics.

In an attempt to resolve this controversy the following experiments were carried out. *E. coli*, K_{12} (1 mg. dry wt./ml.) was incubated for 60 min. at 35° in synthetic medium (ammonium salts and phosphate buffer) containing 50 μc of [^{14}C]glucose (0·2 % final concn.) in the presence and absence of penicillin (100 μg./ml.). Sucrose (20 %, w/v) was added to prevent rupture of spheroplasts. The cells (100 mg. dry wt.) were extracted with hot trichloroacetic acid (5 %, w/v) and lipid solvents. The residue was oxidized with performic acid and hydrolysed with 6N-HCl for 24 hr. at 100°. The hydrolysate in 0·1N-HCl was applied to a column of Zeo-Karb 225 (H^+). DAP and basic amino acids remaining on the column were eluted with 6N-HCl and separated by paper chromatography (butanol–acetic acid solvent for 3 days). DAP was eluted and its specific activity determined. Penicillin depressed incorporation from 335 to 59 counts/min./100 μg. of DAP. The mean inhibition in three experiments was 72 %. The incorporation of glycine and serine were not inhibited by penicillin. Only a trivial part of these amino acids is present as mucopeptide in *E. coli* cell-wall preparations (Mandelstam, 1961).

The time course of incorporation into DAP both in controls and in presence of penicillin was roughly linear. Finally, the inhibition of incorporation by benzylpenicillin was compared with that of 6-[D(−)-α-aminophenylacetamido]penicillanic acid ('penbritin', Rolinson & Stevens, 1961) at a variety of concentrations. At 2 μg./ml. the latter produced about 50 % inhibition; penicillin had no effect at all at this level, and its concentration had to be increased roughly 20-fold to achieve comparable inhibition.

It is apparent from the results that penicillin, and also 'penbritin', have the same effect on mucopeptide synthesis in *E. coli* as in staphylococci, the only essential difference being that much higher concentrations are needed.

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The Catabolism of Bradykinin, Oxytocin, Substance P and Vasopressin by Homogenate of Dog Hypothalamus

By K. C. Hooper. (*Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge*)

The posterior pituitary hormones oxytocin and vasopressin are produced by the hypothalamus (Bargmann & Scharrer, 1951), although a culture of the tissue (Borghese, 1954) did not produce the polypeptides in detectable quantities.

The catabolism of four polypeptides; bradykinin, oxytocin, substance P and vasopressin by homogenate of dog hypothalamus has been examined as a preliminary to developing an *in vitro* system producing oxytocin and vasopressin. The polypeptides are inactivated by homogenate of several areas of brain including the hypothalamus, and evidence suggests that destruction is effected by enzymes. Some properties of these enzymes were described and compared with those of other proteolytic enzymes known to occur in brain.

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Variation in *Carcinus maenas* Hepatopancreas 'Respiring Particle' Activity, with *in vitro* and *in vivo* Changes in Osmotic Pressure

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Osmoregulation in *Carcinus maenas* has been extensively studied. Fredericq (1904) showed changes in blood osmotic pressure when animals are subjected to osmotic stress, and Shaw (1958) demonstrated parallel changes in intracellular muscle fibre fluid tonicity. We have confirmed, with Ramsay's (1949) method, that *Carcinus maenas* behave poikilosmotically with changes in the external medium from 100 to 80 % sea-water strengths, and then homoiosmotically down to 20 % sea water. Blood and muscle fibre fluid always remain virtually iso-osmotic. Subcellular components *in vivo* are therefore subjected, and adapt to, a wide range of osmotic conditions.

An active, purified subcellular 'respiring particle' (mitochondria) preparation has been obtained from the hepatopancreas of *Carcinus maenas* in 0·25M-sucrose (Munday & Thompson, 1961*a*), but this medium is very hypotonic to the normal intra-

cellular tonicity of 0·8 M of our stock crabs. Manometric succinic acid oxidase activity assayed under standard medium conditions (Munday & Thompson, 1961*b*), over a final range of tonicities from 0·2 M to 1·2 M, show peak activities at 0·3 M (58·05 ± 4·25 μl. of O_2/mg. of protein/hr.) and 0·6 M (37·9 ± 3·9 μl. of O_2/mg. of protein/hr.) for particles prepared in 0·25 M- and 0·8 M-sucrose respectively.

Measurements of swelling at 950 mμ under similar conditions to those for manometric assays, show that the 0·25 M and 0·8 M preparations behave osmotically below final concentrations of 0·4 M and M respectively. This suggests the adoption of the preparatory medium tonicity and indicates particle permeability to sucrose. Swelling at each tonicity may possibly be linked with the levels of succinic acid oxidase activity. Two periods of swelling are considered, namely 0–10 min. and 10–35 min.—the period of manometric assay. Below the peak tonicities, decrease in activity is related to increased initial swelling. Above the peak tonicities, there are initially rapid changes in optical density which mimic swelling but presumably record drastic structural changes. At maximum activity there is little initial rapid swelling but later slow and considerable swelling.

It is suggested these results may reflect a mechanism whereby *in vivo* changes in osmotic pressure during osmoregulation can directly regulate 'respiring particle' structure, and hence active cellular adaptation. They also offer an explanation for the frequently observed elevation of respiration in animals under osmotic stress (Schlieper, 1929; Schwabe, 1933; Beadle, 1931; Flemister & Flemister, 1951; Van Weel, 1957).

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